MAPPING COLD TOLERANCE QUANTITATIVE TRAIT LOCI (QTL) IN A *Citrus grandis* x *Poncirus trifoliata* F_1 PSEUDO-TESTCROSS POPULATION USING MOLECULAR MARKERS AND EVALUATION OF HOMOLOGY WITH A RELATED POPULATION MAP

By

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The threat of damage from low temperatures is a great concern in the citrus growing regions of Florida. The related species, *Poncirus trifoliata,* has long been recognized as a desirable source for improved cold tolerance in citrus hybrids. However, the genetic component of this tolerance has not been determined, although it is known that cold tolerance in *Poncirus* is a quantitative trait inherited in a continuous manner in a segregating population. Two genetic linkage maps were produced for a *Citrus grandis* 'DPI 6-4' x *Poncirus trifoliata* 'Rubideaux' F_1 pseudo-testcross population using molecular markers. The map for the *Citrus* parent is the first map based solely upon markers derived from *Citrus* and not mixed with markers from *Poncirus*. Random amplified polymorphic DNA (RAPD) markers proved to be efficient for mapping in this population and several quantitative trait loci associated with tolerance to cold were identified.

In addition, sequence-based PCR markers-including sequence-characterized amplified regions (SCAR), cleaved amplified polymorphic sequences (CAPS), and sequence-tagged sites (STS)-were designed for use in diverse citrus populations. A total of 26 polymorphic, sequence-based PCR markers were identified in the pseudo-testcross population with 24 mapped to 12 linkage groups on the parental maps. In a backcross population, 20/26 were polymorphic with 15 mapped to 7 linkage groups of a published map. Eleven of these markers were located on maps from both populations, allowing for the evaluation of homology between linkage groups on the different maps. The production of these markers was less technically demanding and was achieved in less time than the prevalent marker type used for homology studies between populations.

CHAPTER 1
INTRODUCTION

A plant breeder's ability to solve practical problems hinges on the ability to predict the outcome of various procedures. Such predictions are accomplished through the understanding of the structure of germplasm and the laws governing its structure (Allard 1960). Genetic understanding comes through detailed studies of inheritance in populations. The study of genetics, no matter how basic or complex, is of the utmost importance to a plant breeder. It is to the plant breeder, like anatomy is to a medical doctor, the very foundation of the profession.

Citrus is classified as a cold tender evergreen with a tropical and subtropical origin (Webber 1967). It is grown throughout the world where there are suitable soils and sufficient moisture and where there is not enough cold to kill it (Soost and Cameron 1975). Whereas soil conditions and moisture availability can be modified through cultural and mechanical means, progress in increasing freeze tolerance must be addressed through biological means. Much progress has been made in the area of frost protection, but it typically involves sacrificing the fruit and trying to ensure the survival of the cold sensitive scion. Since the majority of commercial citrus acreage is subject to occasional damaging freezes, cold hardiness of the scion is desirable (Soost and Cameron 1975).

Breeding and cultivar development have become increasingly important to the citrus industry since the late 19th century. Breeding citrus for cold hardiness has been initiated and abandoned numerous times over the last 100 years with little success. The current citrus industry would benefit from cold hardy cultivars. Many problems associated with breeding woody perennials-including long juvenility periods, large plant size, high levels of heterozygosity, reproductive irregularities, and the quantitative inheritance of many economically important characters-have impeded the progress of scion cultivar improvement in citrus (Soost and Cameron 1975). Instances of self-incompatibility, apomixis, and especially polyembryony increase the difficulty of doing genetic studies in citrus and make the lack of genetic information on important traits in citrus not surprising.

Considering the problems involved with breeding citrus, a molecular approach should be useful for the improvement of quantitative traits such as cold hardiness or salinity tolerance in citrus. A molecular approach overcomes the need for multiple generations since differences in phenotype can be related directly to numerous segregating DNA markers. Thus long juvenile periods, fertility irregularities, and the constraints of large plant size can be avoided. In this study, cold tolerance was investigated in an F_1 pseudo-testcross population of *Citrus grandis* (L.) Osb. 'DPI 6-4' x *Poncirus trifoliata* (L.) Raf. 'Rubideaux'. Random amplified-polymorphic DNA (RAPD) markers were generated in order to determine genetic components of the phenotypes in this segregating population via interval mapping. *C. grandis* is a monoembryonic, cold-susceptible citrus species commonly called pummelo. It readily produces viable offspring

when mated with *P. trifoliata,* a cold-tolerant wild relative of citrus. *C. grandis* is susceptible to cold damage at temperatures as high as -6°C, and *P. trifoliata* has been documented to withstand temperatures below -20°C (Yelenosky 1985). The F_1 pseudo-testcross population, which was derived from heterogeneously, heterozygous parents, should exhibit a large range of cold hardiness due to the heterozygosity of the parents and the wide disparity in cold tolerance between them. The isolation of subpopulations of superior and inferior cold-tolerant individuals should also be possible using this population. This would allow the testing of bulked segregant analysis (BSA), a technique used to investigate the genetic differences between individuals in a population at opposite extremes of a frequency distribution.

Finally, toward the utilization of current genetic map information published from the backcross population of *Citrus grandis* 'Thong Dee' x (*Citrus grandis* 'Thong Dee' x *Poncirus trifoliata* 'Pomeroy') (Cai et al. 1994), anchor markers were designed from sequence obtained from cloned *Citrus* and *Poncirus* DNA sequences and the Genbank EST database. PCR primers were designed to amplify specific DNA sequences in order to locate polymorphisms that can be utilized in multiple related populations. These anchor markers common to both populations were used to identify regions of colinearity and presumed homology between the genetic maps from the different populations to present a more complete genetic map for *Citrus* and *Poncirus*.

CHAPTER 2
LITERATURE REVIEW

The threat of damage from low temperatures is a concern in the majority of the citrus-growing regions of Florida. Most citrus growing areas experience occasional damaging freezes ranging from light, fruit-damaging freezes to severe, tree-damaging ones. For regions susceptible to low temperatures, much progress has been made in the area of frost protection. Tree wraps became available in the 1950s as a means to protect young tree trunks and have been shown to be effective in mild freezes (Rose and Yelenosky 1978). The use of under-tree sprinklers has become a common practice for the protection of small trees and the scion/rootstock union in larger trees. Temperature increases of > 7°C were obtained with high-volume sprinklers during radiative freezes in the lower canopy of several citrus cultivars and increased harvest of fruit after a freeze (Wilcox and Davies 1981). However, these methods are limited to young trees or graft unions on larger trees because complete coverage of large trees causes severe structural damage due to the weight of the ice which forms. The use of sprinklers is also subject to fresh water availability, which is becoming increasingly limited. These methods typically involve sacrificing the fruit and trying to ensure the survival of a cold-sensitive scion cultivar. Thus, despite progress in protecting trees from freeze damage via mechanical

means, ultimately biological methods will be necessary to produce cold-tolerant citrus varieties.

Breeding and cultivar development have become increasingly important to the citrus industry since the late 19th century. The first organized *Citrus* breeding program was initiated in 1893 by the United States Department of Agriculture (USDA) employees W.T. Swingle and H.J. Webber in Florida (Cooper et al. 1962). After a severe freeze in 1894-95 Swingle began using the cold-resistant trifoliate orange (*Poncirus trifoliata*) as a parent in crosses to produce cold-hardy hybrids. In 1908, the USDA in Orlando, Florida produced more trifoliate orange crosses, yet no individual from this population was obtained with good cold hardiness and good fruit quality. However, the 'Troyer' citrange originated from this population and became an important rootstock cultivar (Soost and Cameron 1975). In 1942, another set of hybrids using the grapefruit x mandarin hybrids 'Clementine' and 'Orlando' as parents was produced in Orlando, with more produced in 1948. Out of these, some mandarin-type cultivars with increased cold-tolerance were released including, 'Robinson', 'Osceola', 'Lee', and 'Nova' (Soost and Cameron 1975). Thus, a few scion cultivars with improved cold resistance have been produced, but few of commercial importance are in cultivation today.

Cold Stress

Low temperatures limit the productivity of and geographical distribution of plants in large areas of the world (Alberdi and Corcuera 1991). Low temperature decreases the biosynthetic activity of plants and normal physiological functions and can cause

significant damage leading to the death of the plant (Levitt 1980). Many plants have the capacity to survive low temperatures. However, maximum capacity for most plants is dependent upon a period of acclimation prior to the low temperature during which various physiological changes occur. Carbohydrates, amino acids, proteins, as well as other compounds accumulate, and membrane alterations occur that increase the ability of the plant to survive low temperatures (Alberdi and Corcuera 1991). In citrus, acclimation involves increases in sugars and amino acids and often a decrease in starch in the leaves (Yelenosky 1982).

Current citrus varieties grown in Florida are susceptible to occasional severe freezes. Since the 1820's, at least nine very destructive freezes have been recorded (Barrett 1981). In the 1962 freeze, 25% of Florida's citrus trees were killed (Barrett 1981). The current USDA cold-hardy citrus breeding program utilizes improved cold tolerance seen in some citrus relatives (Barrett 1981). *Poncirus trifoliata* is a cross-compatible relative that has been documented to withstand temperatures in below -20°C (Yelenosky 1985). It has been hybridized to cultivars with superior fruit quality and poor cold tolerance such as *Citrus grandis*, which has mild-flavored fruit and is susceptible to cold damage at temperatures as high as -6°C (Yelenosky 1985). The identification of genomic regions associated with cold tolerance in *Citrus grandis* and the closely related species, *Poncirus trifoliata,* is a step in locating and directly manipulating genes responsible.

Genetic Mapping

The accumulation of genetic data is a crucial step in the process of plant breeding (Allard 1960). An extensive genetic database allows for efficient planning of a program and increases the probability of success. Tree fruit crops are generally not conducive to this type of information gathering due to long juvenility periods, large plant size, and reproductive irregularities (Monet 1989).

Historically, genetic maps were based upon the segregation of single genes controlling distinct morphological or qualitative traits. Traits such as peach fruit flesh-color (Connors 1923), flower type in peach (Bailey and French 1942), and resistance to citrus tristeza virus in *Citrus* (Yoshida 1985, 1993; Gmitter et al. 1996) are determined by the effect of alleles at a single locus. By identifying enough single gene traits in a species, one can build a map of its genome based on the segregation of these traits in relation to each other. However, it is difficult to accumulate sufficient numbers of such markers in a single population. Molecular markers have made mapping genomes of diverse species possible by making the generation of large numbers of segregating, phenotypically-neutral markers a reality.

Restriction fragment-length polymorphisms (RFLP) were the first molecular DNA markers used by population biologists. An RFLP results when variation in restriction enzyme cleavage sites is detected by Southern blot hybridization, a procedure where DNA fragments, separated by gel electrophoresis, are transferred to a nitrocellulose or nylon filter to be probed by known DNA sequences (Southern 1975). The difference in DNA fragment size due to differences in digestion can then be detected. RFLPs are

considered the standard for reliability by most researchers. They are codominantly inherited, allowing greater information for each marker locus to be gathered. Also, since the specific probe used to identify the RFLP is known, the markers identified in different populations using the same probe can be equated with a greater degree of certainty than those produced with other marker types such as random-amplified polymorphic DNA (RAPD) markers.

Williams et al. (1990) and Welsh and McClelland (1990) proposed using arbitrary primers to produce a new type of DNA polymorphism for use as genetic markers using the polymerase chain reaction (PCR). This process of arbitrarily-primed PCR (AP-PCR) produces dominant and repeatable polymorphisms termed random-amplified polymorphic DNA (RAPD). RAPD markers allow for the production of a genetic linkage map quickly and relatively inexpensively due to the high levels of polymorphism often seen using this technique. The dominant nature of these markers means that a band on a gel representing an amplified DNA product of specific size is observed in homozygous dominant and heterozygous individuals whereas homozygous recessive individuals are null for that amplified product.

RAPD markers have been used in dozens of plant species to produce genetic markers for cultivar differentiation (Young 1994), genetic mapping (Barua et al. 1993, Cai et al. 1994, Grattapaglia and Sederoff 1994, Hemmat et al. 1994, Kesseli et al. 1994, and Plomion et al.1994), variability assessment (Newbury and Ford-Lloyd 1993), and identification of somaclonal variation (Brown et al. 1993). RAPDs have been used extensively to produce and identify markers closely linked to specific genes of interest.

Michelmore et al. (1991) and Paran and Michelmore (1993) identified RAPDs linked to disease-resistance genes in lettuce, and Barua et al. (1993) identified RAPD markers linked to disease resistance genes in barley. Carlson et al. (1991) studied segregation of RAPD markers in F_1 progeny of conifers. In *Citrus*, RAPDs have provided ample markers for mapping the genome due to the highly heterozygous nature of the genus and related species (Cai et al. 1994).

Population Design

A population used for genetic mapping must show variability for the trait of interest and polymorphism at many marker loci. In forest trees, an innovative population design termed a two-way pseudo-testcross has been used to avoid the problem of long generation times by maximizing segregation of both marker loci and traits of interest (Grattapaglia and Sederoff 1994). In this design, two highly heterozygous individuals that are not closely related are used to make a controlled cross. These individuals should contain many RAPD marker loci that are heterozygous in one parent and homozygous null (i.e., band present and band absent, respectively) in the other parent. In the progeny, segregation should appear in a 1:1 ratio of heterozygous:homozygous null. This allows the parental origin of each marker to be identified and for a map of each parent to be constructed by grouping of markers from each parent for analysis.

Grattapaglia and Sederoff (1994) used the two-way pseudo-testcross population design with RAPD markers to construct a map for *Eucalyptus grandis* and *E. urophylla.* These maps were later used for QTL analysis of micropropagation response, stump

sprouting ability, and rooting ability (Grattapaglia et al. 1995). This type of cross has also been used for mapping in apple (Hemmat et al. 1994) and in conifers (Carlson et al. 1991). A one-way pseudo-testcross was used in *Cichorium intybus*, a self-incompatible perennial chicory with inbreeding depression problems, to produce the first linkage map for the species (Simone et al. 1997). Inbreeding depression is a phenomenon in which offspring of closely related parents so reduced vigor presumably due to an accumulation of deleterious alleles at multiple loci throughout the genome. This population design allowed for mapping in a single generation so this problem was avoided. In a similar population, progeny from an F_1 cross of pine were analyzed for QTL controlling wood production with RAPD markers (Emebiri et al. 1997).

QTL Mapping

Quantitative traits are characterized by continuous variation in a segregating population. Quantitative traits are the phenotypic manifestations for the combined effect of numerous genes. This is in comparison to qualitative traits that are controlled by single genes. Cold hardiness in citrus has been determined to be quantitatively inherited in that no distinct classes of cold tolerance are seen in segregating populations. Rather, a continuous phenotypic variation is seen across a population showing variation for cold hardiness. The loci at which alleles controlling a quantitative trait are located are collectively called quantitative trait loci (QTL). Information on the genetic control for the quantitative inheritance of cold tolerance in *Citrus* and its relatives is unavailable and would be useful for the improvement of citrus varieties.

Mapping of QTL requires a genetic linkage map in order to correlate specific genomic regions with the trait of interest. Genetic linkage maps used to investigate these correlations may only be useful for the particular population that is being studied since some DNA markers, especially RAPDs, are often population specific. RAPD markers allow for the production of a genetic linkage map quickly and relatively inexpensively due to the ability to detect high levels of polymorphism in many species. Data collection with RAPDs has been reported to proceed five times more quickly than with RFLPs (Anderson and Fairbanks 1990). Populations with highly heterozygous parents are especially amenable to genetic mapping using these markers. High levels of heterozygosity increase the likelihood of the detection of polymorphism using RAPD markers. This allows for the efficient construction of a framework of genetic markers with which QTL associated with cold tolerance can be located.

There are several approaches for mapping quantitative traits, including interval mapping, bulked segregant analysis, evaluation of distributional extremes, marker genotype means comparisons (t-test), and one-way analysis of variance (ANOVA) (F-test) of individual markers. Interval mapping has been the most common approach to mapping QTL since the widespread use of molecular markers began. Interval mapping uses an approach called maximum likelihood (Mather 1938) to determine the probability of linkage between a given marker and a known marker or trait (Staub et al. 1996). Maximum likelihood is a statistical procedure designed to choose values for variables that maximize a defined function. After maximization, a ratio of the probability that two loci are linked over the probability that they are not is determined. This is converted to a

log of the odds ratio (LOD) for ease of use (Risch 1992). Using maximum likelihood for analysis of a single QTL flanked by a pair of marker loci allows for the estimation of putative QTL effects at any location within a marker interval based on the means and variances observed in the marker classes and the recombination frequency between markers bracketing a particular interval (Lander and Botstein 1989).

The interval approach takes advantage of information provided by linkage maps that have a relatively high level of genome saturation in the range of markers every 5-20 cM (Staub et al. 1996). This approach has been used on many crop species, including maize (Pe et al. 1993), tomato (Tanksley et al. 1996), and potato (van den Berg et al. 1996), and it can be utilized on any species if map data and quantitative measurements of a trait of interest are available.

Interval mapping is limited, however, by the inability to test unlinked markers and to locate QTL beyond the terminal markers of a given linkage group (Staub et al. 1996). By comparing marker genotype means (t-test) and/or using a one-way ANOVA, one can overcome some of the limitations of examining unlinked markers and incomplete map data. A one-way ANOVA analyzes the variance among the individuals for each marker class to find correlations between individual markers and mean trait values implying a QTL for a trait of interest.

Marker genotype means analysis examines differences between the mean phenotype of a given trait for those individuals with alternative allelic configurations at each marker locus. A t-test or an F-test determines whether there is a statistical difference between these means. In the pseudo-testcross configuration, these two tests are essentially

equal because there are only two classifications for analysis (i.e., the degrees of freedom (df) for analysis is 1). By looking at each marker locus in the population, one can locate clusters of markers on the linkage map that show significant differences between the marker allelic classes. In *Eucalyptus*, a single-factor ANOVA (F-test) was used to locate putative QTL associated with frost tolerance (Byrne et al. 1997). The use of means comparison also allows for the evaluation of markers that are not placed on the linkage map. These markers may be in areas of the genome that show low levels of recombination and/or polymorphism yet may still be important to a trait of interest.

Individual markers can also be evaluated by examining individuals in the distributional extremes of a population for a particular trait. Bulked segregant analysis (BSA) and evaluation of individuals in distributional extremes are techniques used to identify individual markers associated with a phenotype regardless of the level of map saturation. Bulked segregant analysis is a rapid procedure for identifying genetic markers in a specific region of the genome (Michelmore et al. 1991). A comparison of pooled DNA samples from individuals in a single population-segregating for a trait of interest- allows for the identification of markers associated exclusively with that trait.

Individuals in the population are classified into separate bulk subpopulations according to the presence or absence of a particular gene, marker, or trait. Markers that are polymorphic between the bulked samples should be genetically linked to the trait of interest (Michelmore et al. 1991). This allows for the rapid identification of new markers in an area of the genome of interest in order to produce increased marker saturation in that area. It also allows for the continued mapping of a population with a high overall

level of marker saturation. Finally, this method can be used to find markers that do not segregate in the population originally used to generate the genetic map such as for disease resistance genes in tomato for which near isogenic lines were unavailable (Michelmore et al. 1991).

Michelmore et al. (1991) developed and used this method to identify three RAPD markers linked to the downy-mildew-resistance gene in an F_2 lettuce (*Lactuca sativa*) population. They showed that markers can be reliably identified in a 25-centimorgan window on either side of the targeted locus. Barua et al. (1993) used this method with near-isogenic lines of barley (*Hordeum vulgare*) to identify seven RAPD markers linked to a gene for scald or leaf-blotch resistance and to confirm its location on the long arm of barley chromosome 3. Wang and Paterson (1994) concluded that bulk segregant analysis would be useful for tagging QTL with very large effects but may be less useful for identifying loci with lesser effects. More recently, BSA has been used for identifying markers associated with QTL in barley (Chalmers et al. 1993), beet (Pelsy and Merdinoglu 1996), and tomato (Chague et al. 1997).

The evaluation of the individuals making up distributional extremes in a population to locate genetic markers associated with QTL was described by Tanksley (1993). Using this approach, a measure of a quantitative trait is taken for individuals in a large segregating population. Marker analysis is performed only on individuals in the extreme tails of the distribution. If the allele frequency at any molecular marker locus differs significantly between the two extreme subpopulations, it is inferred that a QTL controlling the character of interest is located near the marker. Using BSA and evaluation

of distributional extremes should be helpful in finding markers that show skewed segregation between the extremes yet normal segregation in the population at large or markers showing significantly different trait means between the marker allele classes in the bulk populations.

Sequence-based Markers

RAPD markers exhibit several flaws that make them unreliable for colinearity and homology analysis between related species and populations. First, most RAPD markers show dominant inheritance, which makes the information gleaned from each marker less than that obtained from markers with co-dominant inheritance. Also, the primers most often used to produce RAPD markers are only 10 bases long so that often multiple marker loci are produced with each primer. These markers are usually not genetically linked. Co-dominant markers allow one to identify heterozygous and homozygous individuals. Markers that are locus specific can be used in multiple populations in order to compare homology and colinearity between genetic maps. RFLPs are useful for this purpose but are laborious and expensive to generate.

More useful would be co-dominant sequence-based markers that could be identified using PCR. Many such marker types have been developed, including sequence-characterized amplified regions (SCAR) (Paran and Michelmore 1993), cleaved-amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993), and sequence-tagged sites (STS) (Talbert et al. 1994; Trangoonrung et al. 1992). These marker types use a pair of forward and reverse primers specific to a DNA sequence. This specificity is

due to the length of the primer pairs. Since the DNA code is based upon a four-base alphabet, the possible combinations for these 4 bases are so great when primers reach sufficient length, that the probability of any one specific sequence being randomly repeated in a genome is reduced to a minute level. Multiple site or locus amplification can and does occur in genomes with duplicated sequence, as in many polyploid species. However, the primers are still specific to a particular DNA sequence.

SCAR markers are derived by cloning and sequencing RAPD markers so that primers specific to the sequence can be designed (Paran and Michelmore 1993). The primers usually consist of the RAPD primer followed by the next 8 to 15 bases in the sequence for both DNA strands so that specificity is increased to one locus. By increasing the primer length, one increases the odds against a random primer pair match at a region of DNA sequence unrelated to the sequence which the primers were designed. SCAR markers are sometimes dominantly inherited like the underlying RAPD marker but can be co-dominant in nature, producing bands of different size corresponding to two marker alleles.

The major drawback to SCAR markers is the loss of polymorphism when the RAPD marker is converted to a SCAR. Paran and Michelmore (1993) found that 5 of 8 primer pairs tested for SCAR production were from RAPD sequences where the polymorphisms were apparently due to mismatches at the RAPD primer sites. Fang et al. (1997) found that primers designed from RAPD marker sequences lost the polymorphism when used for SCAR marker production. Xu et al. (1995) also experienced loss of polymorphism when designing primers from RAPD marker sequence. Markussen et al.

(1995) also found that the specificity of SCAR primer sets was not always at a high level presumably due to duplicated or repetitive DNA sequences. They found more than one segregating marker locus with one primer pair.

CAPS markers are derived in several different ways. They can be produced from any type of DNA sequence including cloned RAPDs, expressed-sequence tags (ESTs), sequenced RFLP probes, and genomic DNA clones (Staub et al. 1996). CAPS identify single nucleotide polymorphisms between marker alleles that alter restriction sites for one or more restriction enzymes, enabling the different alleles to be identified because of differential restriction (Konieczny and Ausubel 1993). CAPS have been produced from sequences intended for SCAR markers that did not show polymorphism (Ohmori et al. 1996, Deng et al. 1997). EST sequences (Gilpin et al. 1997; Botella et al. 1997) and genomic clones (Hardtke et al. 1996) have similarly been converted to CAPS for mapping using specific primers and restriction enzyme digestion. However, the production of CAPS markers can be time consuming and lead to little success. With non-polymorphic SCAR fragments in grape, Xu et al. (1995) were unable to find restriction-site polymorphisms even when the amplified fragments were digested with seven different restriction enzymes. One should note that the terminology has not been consolidated in this area and that markers that are produced in a similar manner (i.e., PCR with enzyme digestion) are referred to in several ways including CAPS, SCAR, and EST/STS markers.

STS markers are most commonly derived from sequenced RFLP probes (Blair and McCouch 1997, Larson et al. 1996) or from EST sequences (Gilpin et al. 1997). They are

often similar to CAPS markers in the need for digestion for identification of polymorphism, but size differentiation is sometimes also detected without digestion.

Much progress has been made in the production of genetic linkage maps and in the evaluation and mapping of quantitative trait loci. By combining several approaches for producing molecular markers and for analyzing quantitative data, the probability of locating putative QTL associated with cold tolerance should be increased. From this, a more comprehensive picture of the genetic control of cold tolerance should emerge.

CHAPTER 3

GENETIC LINKAGE MAPPING OF A *Citrus grandis* 'DPI 6-4' x *Poncirus trifoliata* 'Rubideaux' F_1 PSEUDO-TESTCROSS POPULATION WITH RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS

Introduction

Considering the problems involved with breeding citrus scion cultivars-including long juvenility periods, large plant size, high levels of heterozygosity, reproductive irregularities, and the quantitative inheritance of many economically important traits-a molecular approach should be useful for the improvement of quantitative traits such as cold hardiness or salinity tolerance in citrus. Toward that end, several studies of citrus involving molecular markers-including isozymes, restriction fragment length polymorphisms (RFLP), and random amplified polymorphic DNA (RAPDs)-have been published. Torres et al. (1985) characterized isozymes in *Citrus* and *Poncirus*. Durham et al. (1992), Jarrell et al. (192), and Liou et al. (1996) investigated RFLP and isozyme data in characterizing the citrus genome. Eleven genetic-linkage groups were identified using segregation data from a backcross population of *Citrus grandis* 'Thong Dee' x (*Citrus grandis* 'Thong Dee' x *Poncirus trifoliata* 'Pomeroy') (Durham et al. 1992, Liou et al. 1996). Cai et al. (1994) added to the RFLP and isozyme-linkage map using RAPD markers. The eleven linkage groups were reduced to nine linkage groups that could correspond to the number of chromosomes in the haploid genome of *Citrus*, n=9. This

map contains 249 isozyme, RFLP, and RAPD markers spanning 1192 centimorgans (cM) with an estimated 70-80% genome coverage and an average of 7.5 cM between markers. A centimorgan corresponds to 1% crossing over frequency in a segregating population.

Mapping of QTL associated with quantitative traits requires a genetic-linkage map in order to correlate specific genomic regions with the trait of interest. The linkage maps produced using a backcross population have provided a better understanding of the *Citrus* genome and will be useful in future genetic studies. However, in preliminary tests this population was shown to be inadequate for mapping quantitative trait loci (QTL) for cold hardiness due to the lack of significant segregation for the trait. Presumably, the loss of 75% of the *Poncirus* genome, which is the source of increased cold tolerance, through the backcross process, where an offspring is mated with one of the parents in order to introduce inbreeding and reduce heterozygosity, is the root of this lack of segregation. In addition, there was no assay capable of precisely measuring the cold tolerance of individuals in the population for quantitative trait analysis.

A population must be used that shows variability for the trait of interest and useful heterozygosity for mapping. An innovative population design termed a two-way pseudo-testcross has been developed incorporating these features (Grattapaglia and Sederoff 1994). In this design, two highly heterozygous individuals that differ widely for the trait of interest are used to make a controlled cross. These individuals should contain many marker loci that are heterozygous in one parent and homozygous null in the other parent. In the progeny, segregation should appear in a heterozygous:homozygous null ratio of 1:1. This allows the parental origin of each marker to be identified and for a map

of each parent to be constructed by grouping of markers from each parent for analysis. The two-way pseudo-testcross design should provide an ideal QTL mapping population for *Citrus* with the appropriate parents.

Yelenosky et al. (1993) evaluated seedlings from a cross of *C. grandis* x *P. trifoliata* for cold tolerance at -6.7°C and found a wide range of cold tolerance. *Citrus grandis* and the related species *Poncirus trifoliata* are heterozygous species that differ widely in their tolerance to cold and for alleles at marker loci. An F_1 pseudo-testcross population using these parents should produce a segregating population suitable for linkage mapping and QTL analysis for cold tolerance.

Material and Methods

Plant Material

The segregating population consisted of 500 seedlings of a *C. grandis* 'DPI 6-4' x *P. trifoliata* 'Rubideaux' F_1 pseudo-testcross (pummelo x trifoliate orange) hybridization made at the USDA Citrus Research Center in Orlando, Florida. Seeds from the fruit of this cross were extracted and dried before planting. The seed coat was removed from each seed to improve germination speed and uniformity. The peeled seeds were planted in the greenhouse in a soilless medium in 1" x 1"x 2" six-pack seedling trays. The seedlings were grown for approximately 3 months before transfer to 2" x 10" 'conetainers' in racks of twenty. Trees were fertilized monthly with 300 ppm of a water soluble commercial fertilizer (Peters 20-20-20 with micro nutrients).

A random subpopulation of 61 individuals was used to produce the maps for interval mapping of QTL associated with cold tolerance. In the 'DPI6-4' x 'Rubideaux' cross, the pummelo parent was considered the null parent for cold tolerance in the pseudo-testcross since it is very sensitive to freezing temperatures and was not expected to contribute to increased cold tolerance in the progeny. The trifoliate orange is an apomictic line that is very tolerant to low temperatures and should retain a high degree of heterozygosity at all loci, including those controlling cold hardiness. The level of heterozygosity in the pummelo parent was in question and was determined during marker production. Preliminary tests showed 100% damage of the seedlings of the pummelo parent at -9°C, while the nucellar seedlings of the trifoliate orange parent displayed 0% damage at -15°C. Thus, it was expected that the F_1 pseudo-testcross population would show a wide range of variability for cold tolerance because of the high levels of heterozygosity in each parent and the wide disparity in their tolerance to cold,.

DNA Isolation

Genomic DNA was isolated as described by Durham et al. (1992) and modified for 1.5 mL microfuge tubes. Young shoot material was used when possible for highest yields, but mature leaf material produced adequate yields when necessary. Approximately 100 mg of tissue was ground to a powder in liquid nitrogen using Teflon maceration tips in 1.5 ml microfuge tubes. Then 700 μl of extraction buffer [Tris-HCl (pH 8.0), 50 mM Na_2EDTA (pH 8.0), 500 mM NaCl, 10 mM β-mercaptoethanol, 3% SDS] was added to the tissue with additional grinding. The tubes were then incubated at 65°C for 10

minutes. One volume of a 1:1 phenol:chloroform mixture was added and mixed by inverting, and then the tubes were centrifuged at 12k rpm in a bench-top microcentrifuge for 10 minutes. The aqueous phase was removed to a new tube, and 1 volume of the 1:1 phenol:chloroform mixture was added and mixed by inverting. The emulsion was centrifuged at 12k rpm for 5 minutes, and the aqueous phase was transferred to a new tube. One volume of chloroform was added and mixed by inverting. The emulsion was centrifuged at 12k rpm for 5 minutes, and again the aqueous phase was transferred to a new tube. Then 10 μl of 5M Na-acetate and 0.6 volumes of ice-cold iso-propanol was added to the solution to precipitate the DNA. The tube was centrifuged at 12k rpm for 10 minutes and the liquid poured off the pellet, which was then washed with 1 ml of 75% ethanol and allowed to dry on the bench top for 30 minutes. The DNA was then hydrated in 100 μl of Tris-EDTA buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0)] (Sambrook et al. 1989). The DNA concentration was determined using a spectrophotometer, and a diluted DNA stock of 2 ng/μl was made for DNA amplification.

Primer Screening and DNA Amplification

Random primers (Operon Technologies) were initially screened to detect DNA polymorphisms using the two parents and 6 progeny. Primers used for mapping previously in *Citrus* (Cai et al. 1994) were first screened to increase the efficiency of marker production. An additional 60 primers from the B, C, and R kits (Operon Technologies) were screened to produce additional markers. In total, 180 primers were screened for polymorphism from the A, B, C, E, F, G, Q, R, and T primer kits. DNA

amplification was done following the PCR conditions of Cai et al. (1994) except that 50 μl instead of 25 μl reactions were used. Each 50 μl reaction consisted of buffer [50mM KCl, 10 mM Tris-HCl (pH 9.0), 1% Triton X-100] (Promega), 2 mM $MgCl_2$, 0.8 mM dNTPs (200 μM of each dNTP), 0.4 μM primer, 2 U of Taq polymerase (Promega), 30 ng of genomic DNA and water to volume. Amplification was done on two MJ PTC-100™ thermocontrollers with 96 wells (MJ Research, Inc.). One had a heated lid and did not require overlay of the reactions with mineral oil and the other required oil overlay. The following conditions were used: 1 minute at 93°C, 1 minute at 35°C, and 2 minutes at 72°C for 42 cycles. A final extension step at 72°C for 10 minutes concluded the amplification. The amplification products were separated in 2% agarose gels with TAE buffer (Sambrook et al. 1989) by electrophoresis in a wide mini sub™ cell (Bio-Rad) and detected by ethidium bromide staining (Figure 3-1) with an IS-1000 Digital Imaging system (Alpha Innotech Corp.)

Linkage Analysis

Primers identified through the screening process were used to amplify DNA from the subpopulation of progeny for mapping. All 61 individuals were screened with 37 primers and half of the subpopulation was screened with 34 primers to speed up the marker production. The polymorphic markers were grouped based on their parental origin and goodness of fit to the hypothesized segregation ratio of 1:1 was determined by chi-square analysis. A core map of markers fitting the expected 1:1 ratio was made using the JoinMap computer program (Stam 1993) at linkage group LOD threshold of 3.0.

Additional markers unlinked at LOD 3.0 or showing skewed segregation were placed on the map at LOD $\geq$ 2.0 to fully utilize the marker data. Markers were named with OP followed by a letter and a 2 digit number (for their Operon kit origin) and an additional 3 digit number indicating the size of the amplified product with a factor of ten base pairs.

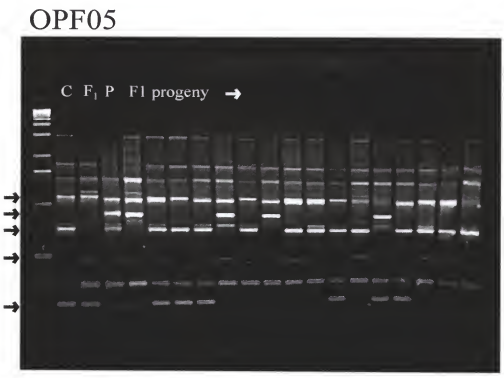

Figure 3-1: Random-amplified polymorphic DNA (RAPD) profile for primer OPF05.The first lane contains DNA fragments of known size for size comparison. The second lane contains amplified fragments from DNA of the *Citrus* parent, the third lane contains fragments from a control F_1 plant for which the RAPD profile is known, and the fourth lane has fragments generated from the *Poncirus* parental DNA. The remaining lanes contain fragments amplified from DNA of individuals in the pseudo-testcross population. Polymorphisms are indicated with arrows.

Results

A total of 71 primers identified as producing usable polymorphisms were screened with the mapping subpopulation (Table 3-1). A total of 284 RAPD markers were identified (Table 3-1) for mapping both the *P. trifoliata* and *C. grandis* genomes, including 26 markers skewed between a 2:1 and 1:1 ratio and 29 markers skewed beyond the range of usefulness. For the *Citrus* parent, 119 markers showing 1:1 segregation were analyzed for linkage mapping, and 109 unskewed markers were analyzed to produce the *Poncirus* parental map. A core map for *Citrus* was made consisting of 13 linkage groups and 3 sets of paired markers. For *Poncirus,* a core map of 10 linkage groups and 2 sets of paired markers was made. Further analysis using JoinMap (Stam 1993) at a LOD of ≥ 2.0, including the skewed markers, coalesced each map into 9 linkage groups. The *Citrus* map consists of 105 markers with a total map distance of 655 cM and an average distance of 6.2 cM between markers (Figure 3-2). Eight of the skewed markers were mapped with four mapped to the end of linkage groups (Figure 3-2). The *Poncirus* map consists of 88 markers with a total map distance of 587 cM and an average distance of 6.7 cM between markers (Figure 3-3). Twelve of the skewed markers were mapped with 11 of these mapping to the end of 3 linkage groups (Figure 3-3).

Table 3-1: Random primer kits screened and number of polymorphic RAPD markers identified and mapped.

Primer Kit[a]	Number of Primers Used	Number of Markers Identified	Number of Markers Mapped
OPA	8	35	31
OPB	11	38	14
OPC	13	56	42
OPE	12	51	30
OPF	5	23	19
OPG	6	28	19
OPQ	1	4	3
OPR	12	35	21
OPT	3	14	14
Total	**71**	**284**	**193**

a- From Operon Technology in kits of 20

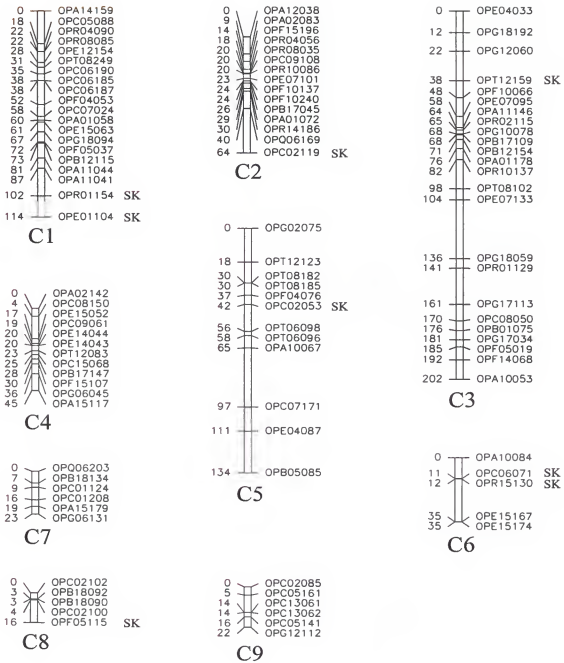

Figure 3-2: Linkage map for *Citrus grandis* produced with RAPD markers. The map consists of 105 markers with a total map distance of 655 cM, averaging 6.2 cM between markers. Skewed markers are indicated with the letters SK.

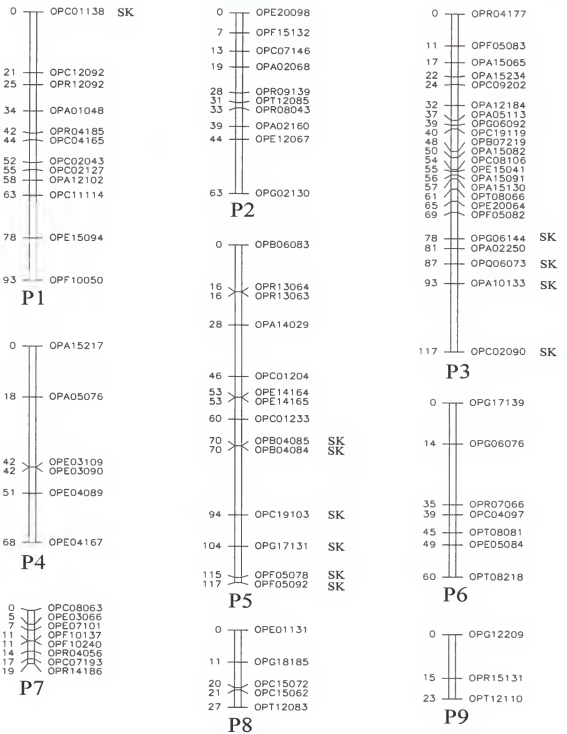

Figure 3-3: Linkage map for *Poncirus trifoliata* produced with RAPD markers. The map consists of 88 markers with a total map distance of 587 cM, averaging 6.7 cM between markers. Skewed markers are indicated with the letters SK.

Discussion

The pseudo-testcross population design has proven to be excellent for mapping *C. grandis* and *P. trifoliata.* The wide cross between *C. grandis* 'DPI 6-4' x *P. trifoliata* 'Rubideaux' produced ample polymorphisms using RAPD markers to construct a linkage map of both *Poncirus* and *Citrus*. RAPD markers have been used for dozens of plant species to produce genetic markers for cultivar differentiation (Young 1994), genetic mapping, (Barua et al. 1993, Cai et al. 1994, Grattapaglia and Sederoff 1994, Hemmat et al. 1994, Kesseli et al. 1994, and Plomion et al.1994), variability assessment (Newbury and Ford-Lloyd 1993), and identification of somaclonal variation (Brown et al. 1993). RAPDs have been used extensively to produce and identify markers closely linked to specific genes of interest. Michelmore et al. (1991) and Paran and Michelmore (1993) identified RAPDs linked to disease-resistance genes in lettuce. Barua et al. (1993) identified RAPD markers linked to disease-resistance genes in barley. Carlson et al. (1991) studied segregation of RAPD markers in F_1 progeny of conifers. In *Citrus*, RAPDs have provided ample markers for mapping throughout the genome due to the highly heterozygous nature of the genus and related species (Cai et al. 1994). The number of RAPD markers produced for this population was 4 to 5 times greater than the isozyme and RFLP markers developed for the related backcross population (Torres et al. 1985, Durham et al. 1992, and Liou et al.1996). The level of polymorphism in the pummelo parent was higher than anticipated, based on the level seen for RFLP and isozyme markers in the previous studies (Torres et al. 1985, Durham et al. 1992, Liou et al. 1996). This may be because the pummelo cultivar in the pseudo-testcross population has a

higher level of heterozygosity than the pummelo parent used in previous studies because it is a selection in a current breeding program. Also, the nature of RAPD markers increases the likelihood of identifying single-base DNA polymorphisms in the genome that may be difficult to observe with other marker systems. These types of polymorphisms would be less likely to be identified with RFLP technology because they would have to be located within DNA sequences susceptible to restriction-enzyme digestion. Single base DNA polymorphisms would also be difficult to identify in isozyme loci because differences in the amino acid sequence of the enzymes would not necessarily occur from a single base change.

There were a large number of RAPD markers identified that could not be placed on either of the linkage maps. This phenomenon has been seen before (Cai et al. 1994) and may be due to various causes. RAPD markers are prone to inconsistency because many of the polymorphisms are due to the single base differences within the primer annealing site. Since the primer annealing temperature for RAPD reactions is low, mismatches may not stop the amplification reaction thus producing spurious or inconsistent markers. Some polymorphisms may also be from extra nuclear genomes; these would not segregate normally. Some of the markers could also be from regions in the genome with low levels of polymorphism and crossing over, such as near the centromere and telomere of each chromosome. The skewed markers near the end of some linkage groups may be due to recombination interference of the telomeric region of the chromosomes. Other skewed markers within the linkage groups could be a result of interference from the centromeric regions.

The *C. grandis* map is the first true map of *Citrus* as it was produced with polymorphisms originating solely from *Citrus.* Earlier maps mostly mapped polymorphisms originating from the related species *Poncirus trifoliata.* Grattapaglia and Sederoff (1994) used the two-way pseudo-testcross population design with RAPD markers to construct a map for *Eucalyptus grandis* and *E. urophylla.* These maps were later used for QTL analysis of micropropagation response, stump sprouting ability, and rooting ability (Grattapaglia et al. 1995). This type of cross has also been used for mapping in apple (Hemmat et al. 1994) and in conifers (Carlson et al. 1991). A one-way pseudo-testcross was used in *Cichorium intybus*, a self-incompatible perennial chicory with inbreeding depression problems, to produce the first linkage map for the species (Simone et al. 1997). In a similar population, progeny from an F_1 cross of pine were analyzed for QTL controlling wood production with RAPD markers (Emebiri et al. 1997). The maps produced in this study provided a good framework for doing QTL analysis for cold tolerance in this population. The 9 linkage groups on each map and the level of saturation indicates a good coverage of the genome and should allow for the identification of QTL associated with cold tolerance.

CHAPTER 4
MAPPING QUANTITATIVE TRAIT LOCI (QTL) ASSOCIATED WITH COLD TOLERANCE IN A *Citrus grandis* 'DPI 6-4' x *Poncirus trifoliata* 'Rubideaux' F_1 PSEUDO-TESTCROSS

Introduction

A quantitative trait is characterized by continuous variation over a segregating population. Quantitative traits are the phenotypic manifestation of the combined effect of numerous genes. This is in comparison to qualitative traits that are controlled by single genes. Many economically important traits in crop species, such as yield components, tolerance to environmental stresses (e.g., low or high temperatures or excess salt), and tolerance to pests, are often quantitatively inherited. As such, it is difficult to separate the genetic effect of each gene on the phenotype of a particular trait. The places where alleles controlling a quantitative trait are located are collectively called quantitative trait loci (QTL). Mapping QTL is an attempt to separate the individual effect of each gene that influences a given trait.

Since *Citrus* is a cold-tender evergreen, it can only be grown between 40° N and 40°S latitude if temperature, soil, and moisture requirements are met. Soil conditions and moisture availability can be modified through cultural and mechanical means, but future progress in increasing freeze-tolerance must be addressed through biological means. The progress that has been made in the area of frost protection involves sacrificing the fruit to

try to ensure the survival of the cold-sensitive scion. Since the majority of commercial citrus acreage is subject to occasional damaging freezes, cold hardiness of the scion is desirable (Soost and Cameron 1975). Breeding and cultivar development have become increasingly important to the citrus industry since the late 19th century. However, there are many problems associated with breeding woody perennials, including long juvenility periods, large plant size, high levels of heterozygosity, reproductive irregularities, and the quantitative inheritance of many economically important characters. These problems have impeded the progress of cultivar improvement in citrus (Soost and Cameron 1975). Instances of self-incompatibility, apomixis, and polyembryony increase the difficulty of doing genetic studies in citrus because hybridization becomes difficult so that suitable populations for genetic studies can not be produced. This makes the lack of genetic information on important traits in citrus not surprising.

Breeding citrus for cold hardiness has been initiated and abandoned numerous times over the last 100 years. However, the lack of genetic information available to breeders concerning the inheritance of cold tolerance has impeded the progress of improvement. Cold hardiness in citrus is quantitatively inherited which means no distinct classes of cold tolerance are seen in segregating populations. Rather, a continuous phenotypic variation is seen across a population showing variation for cold hardiness. Thus, few scion cultivars with improved cold resistance have been produced. The current citrus industry would benefit from cultivars with improved cold resistance.

Considering the problems involved with breeding citrus, a molecular approach should be useful for the improvement of quantitative traits such as cold hardiness in

citrus. A population varying widely in cold tolerance, such as the hybrid population of *C. grandis* 'DPI 6-4' x *P. trifoliata* 'Rubideaux' F_1 studied here, can be evaluated using random amplified polymorphic DNA (RAPD) markers in order to locate putative QTL associated with cold tolerance.

C. grandis, also known as pummelo, is a cold-susceptible citrus species. It readily produces viable offspring when mated with *P. trifoliata,* a cold-tolerant wild relative of citrus. This population should exhibit a large range of cold-hardiness variability due to the heterozygosity of the parents and the wide disparity in cold tolerance between them. *C. grandis* is susceptible to cold damage at temperatures as high as -6°C, and *P. trifoliata* has been documented to withstand temperatures below -20°C (Yelenosky 1985). The isolation of subpopulations of superior and inferior cold-tolerant individuals for distributional extremes evaluation and bulked segregant analysis should also be possible using this population.

The variation in cold tolerance in this population could be due to the effects and interactions of many genes or due to a large environmental effect upon one or a few genes. It could also be due to the major effects of one or a few genes with other genes contributing lesser effects. Tanksley (1993) has shown that this is surprisingly often the case with quantitatively inherited traits. Situations with one or a few genes contributing major effects are the most favorable for marker assisted selection of QTL since the number of markers necessary to follow is reduced.

In order to locate QTL related to cold tolerance in *Citrus*, an assay capable of accurately measuring the level of tolerance in individuals in a population is critical. One

must be able to measure the quantitative trait accurately to be able to identify genomic regions associated with the variability. Several methods have been used to measure cold tolerance in many crops, including electrolyte leakage of leaf tissue (Tignor et al. 1998), whole plant freezing (Yelenosky et al. 1993), leaf tissue chlorophyll fluorescence (Hetherington et al. 1983), and stem section freezing (Zatylny et al. 1996). Soost and Cameron (1975) state that the most reliable procedure to evaluate cold hardiness in *Citrus* is to determine injury following exposure of whole plants to freezing conditions. Yelenosky (1979, 1990) and Yelenosky et al. (1993) have used whole plant freeze tests to evaluate cold tolerance of various *Citrus* and *Citrus* hybrid trees. This method has been used successfully to rank individuals in similar populations in accordance to their cold tolerance and should be effective for this population for mapping QTL associated with cold tolerance.

There are several approaches for mapping quantitative traits, including interval mapping, bulked segregant analysis, evaluation of distributional extremes, marker genotype means comparisons (t-test), and one-way analysis of variance (F-test) of individual markers. Interval mapping has been the most common approach to mapping QTL since the widespread use of molecular markers began. Due to the incredible volume of calculations required to do interval mapping, computer programs such as MapMaker-QTL (Lander et al. 1987) and MapQTL (Van Ooijen and Maliepaard 1996) have been designed to simplify mapping QTL with molecular markers.

The interval approach is limited, however, by the inability to test unlinked markers and to locate QTL beyond the terminal markers of a given linkage group (Staub

et al. 1996). By comparing marker genotype means (t-test) and/or using a one-way ANOVA, one can overcome the limitations of examining unlinked markers and incomplete map data. Bulked segregant analysis (BSA) and evaluation of individuals in distributional extremes attempt to identify individual markers associated with a phenotype regardless of the level of map saturation.

The evaluation of the individuals making up distributional extremes in a population to locate genetic markers associated with QTL was described by Tanksley (1993). Using this approach, a measure of a quantitative trait is taken for individuals in a large segregating population. Marker analysis is performed only on individuals in the extreme tails of the distribution. If the allele frequency at any molecular marker locus differs significantly between the two extreme subpopulations, it is inferred that a QTL controlling the character of interest is located near the marker. Using BSA and distributional extreme evaluation should be helpful in finding makers that show skewed segregation between the extremes yet normal segregation in the population at large or markers showing significantly different means between the marker allele classes in the bulk populations.

Linkage map and marker data previously generated for this population was used in conjunction with quantitative-phenotypic measurements to identify putative QTL in this population. BSA was also used to find markers associated with QTL for cold tolerance in combination with the evaluation of distributional extremes. The level of saturation of the map was dependent upon the level of polymorphism observed and all phases of the investigation proceeded concurrently so the level of success met by each method could

not be anticipated. By using many analysis procedures, the hope was to utilize all the data generated to its fullest in order to locate QTL associated with tolerance to low temperatures.

Materials and Methods

Freeze Testing

The plant material was the same as described in Chapter 3 for mapping with RAPD markers. Seedlings were grown for approximately 18 months before testing for cold tolerance began. An acclimation protocol was followed for the seedlings starting with 21°C, 12 hr days and 10°C nights for two weeks followed by 15°C days and 4°C nights for two additional weeks (Yelenosky et al. 1993). This was done in a walk-in growth chamber equipped with high intensity metal halide growth lamps. After four weeks of acclimation, freeze testing proceeded.

The general freeze test procedure followed that used by Yelenosky et al. (1993) for pummelo x trifoliate orange hybrids. The freeze tests were performed in a walk-in cooler modified for low temperatures and controlled by the GEC134S Precision Temperature Measurement and Control System (Gaffney Engineering, Gainesville, Florida). This system precisely controls the rate of cooling in the chamber and has 13 additional thermocouples to measure the temperatures at various positions in the chamber. Conetainer trays containing 20 seedlings were placed in insulated boxes molded to the shape of the conetainers, and the soil surface was covered with a 1 inch layer of vermiculite to prevent damage to the root system. The root system of a citrus tree does

not acclimate and is vulnerable to freezing temperatures. Five trays of 20 seedlings were tested simultaneously.

The freeze test began with a holding period of 2 hours at 2°C followed by a ramping of -1°C/hour down to the desired low temperature. The trees were held for 4 hours at the low temperature, which was then ramped back up to 5°C at +1°C/hour . Once this temperature was reached, the trees were removed to the greenhouse for damage assessment. Percent dieback was measured approximately 1 month post freezing when new buds broke and began to grow from below damaged stem regions. Regrowth for 3 months after bud break was allowed before further freeze testing.

QTL Mapping

Interval mapping was done using MapMaker-QTL (Lander et al. 1987) and MapQTL (Van Ooijen et al. 1996) with a LOD score threshold of 2.0 to locate putative QTL. MapQTL calculates a LOD score for all marker intervals on the map. For means comparison, a paired data t-test was performed according to the procedures of Ott (1988) with a significant P value ≤ 0.05 set. One way ANOVA was performed by MapQTL showing all markers with significant differences between the marker allele classes with a $P \leq 0.05$ (Van Ooijen et al. 1996).

Results

Citrus and its relatives require an acclimation period to reach their maximum cold-tolerance level, as do most woody perennials. Acclimation corresponds to a period

of time during which the tree normally prepares for winter temperatures through a series of membrane and protein changes and a transition into a state of dormancy. In this study, the acclimation procedure described by Yelenosky et al. (1993) for open pollinated pummelo x trifoliate orange hybrids was used. The freeze testing followed the procedure of Yelenosky et al. (1993). This entailed putting the trees into a chamber where the temperature could be lowered at a controlled constant rate with a holding period at the low temperature. Yelenosky (1979) has determined that -6.7°C is a temperature at which most *Citrus* scion cultivars show discernable freeze damage. For this population, enhanced cold tolerance was expected so that testing at various temperatures was required to determine temperatures at which exceedingly cold-sensitive and superior cold-tolerant individuals could be identified. Freezes at progressively lower temperatures with parental seedlings and a random sample of 20 trees in the pseudo-testcross population were carried out at -6.7°C, -9°C, -12°C, and -15°C to determine optimal temperatures for generating quantitative data that would distinguish individuals in the population from each other.

It was determined that -9°C could be used to identify individuals especially susceptible to cold, and that -15°C could be used to identify the most tolerant individuals. Data from these tests was used for the differentiation of bulk populations for use in BSA and distributional extremes analysis. In addition, the stem dieback at both temperatures was measured for each individual in the random mapping population for interval mapping and mean comparison testing. The pseudo-testcross trees were first frozen at -9°C in 5 freeze tests, allowed to regrow, and then retested at -15°C.

The freeze test to -9°C produced a damage curve skewed towards individuals showing no stem dieback (Figure 4-1). Only 71 of 403 (18%) of the trees tested had any stem dieback at this temperature. Freezing at -15°C produced damage in a more normal distribution skewed toward those individuals showing > 95% damage (Figure 4-1). Of the trees tested at -15°C, 49 of 442 (11%) showed >95% stem dieback, and only 30 of 442 (7%) had dieback of less than 20%. The mean stem dieback at -9°C for the cold-sensitive parent, *C. grandis*, was >95% for the 40 individuals tested, and the mean stem dieback at -15°C for the cold-resistant parent, *P. trifoliata* was <10% for the 40 individuals tested. This demonstrates the huge difference in cold tolerance between the parental types. No parental or F_1 seedlings were killed during the freeze tests as even those with 100% dieback had regrowth from below the stem level protected by vermiculite. This indicates that the method of protecting the root zone from freezing temperatures was successful.

The 10 trees most cold damaged at -9°C were selected for the freezing susceptible bulk and had a mean of 34% stem dieback at this temperature. The ten trees most resistant to freezing at -15°C were selected for the freezing resistant bulk and had mean stem dieback of 7.4% at this temperature. The cold-sensitive trees all had significant stem dieback at -15°C with an average of 69% dieback. Of the cold-tolerant bulk, only 1 tree showed any stem dieback at -9°C. One hundred random primers (Operon Technologies) were screened on the bulked DNA from the selected individuals to identify markers associated with the distributional extremes. Markers putatively polymorphic between the

bulked samples, but monomorphic within the bulks, were then genotyped in the mapping subpopulation for means comparisons.

The mean dieback in the mapping subpopulation at -9°C was 3.8% with 10 of 55 (18%) tested individuals showing significant damage. The mean dieback at -15°C was 56.6% with 5 of 56 (9%) showing >95% damage. These numbers closely resembled those of the whole population.

Interval mapping using the quantitative data and segregation data from the mapping subpopulation produced significant LOD scores using both MapMaker-QTL (Lander et al. 1987) and MapQTL (Van Adjoin et al. 1996). With the markers originating from *C. grandis* (i.e. the band was present in *C. grandis* and not in *P. trifoliata*) , two overlapping intervals correlated to damage at -9°C were identified on linkage group C1 with peak LOD scores of 2.25 and 2.45 using MapQTL and MapMaker-QTL, respectively (Figure 4-2). Another interval associated with damage at -15°C was located on linkage group C5 with a peak LOD score of 1.8 (Figure 4-2) using MapQTL.

With the markers originating from *P. trifoliata,* a minor QTL was located with MapQTL on linkage group P1 with a peak LOD score of 2.5 associated with damage at -15°C (Figure 4-3). On linkage group P5, three overlapping intervals associated with damage at -9°C were located with MapMaker-QTL with peak LOD scores of 3.25, 19.46, and 17.5 (Figure 4-3), and two overlapping QTL intervals in the same region with LOD scores of 1.8 and 17.4 (Figure 4-3) were identified using MapQTL.

Mean comparisons and one way ANOVAs produced similar results in that many of the markers showing significant association with quantitative data were identified with

both methods. On the *Citrus* map, 21 markers with significantly different means for the marker present and absent classes were mapped with 6 associated with damage at the higher temperature and 15 with damage at the lower temperature (Figure 4-2). Eleven markers were located with a one-way ANOVA and 9 of these corresponded to markers located through mean comparison (Figure 4-2). Four were associated with damage at the higher temperature and 7 with the lower temperature. On the *Poncirus* map, 19 markers were identified using mean comparison analysis with 10 associated with damage at -9°C and 10 with damage at -15°C (1 was associated with both) (Figure 4-3). Using a one-way ANOVA, 4 markers associated with damage at -9°C and 9 associated with damage at -15°C were identified (Figure 3). Eleven of these markers were located with both methods.

All of the intervals mapped were associated with clusters of markers identified through mean comparisons and one-way ANOVA (Figures 4-2 and 4-3). On the Citrus map there was also a cluster of markers with significantly different means between the marker present and marker absent classes on the end of linkage group C7 (Figure 4-2) and on the *Poncirus* map at the top of linkage group P3 (Figure 4-3).

With bulk segregant analysis, 11 primers identified putative markers associated with the distributional extremes. The individuals from each bulk population were genotyped for these 11 primers and 17 markers segregating between the groups were analyzed for differences in mean damage at both temperatures between the band present and absent classes. Of these, 8 markers were identified with significantly different means between the marker present and absent classes in the bulk populations (Table 4-1) and 5

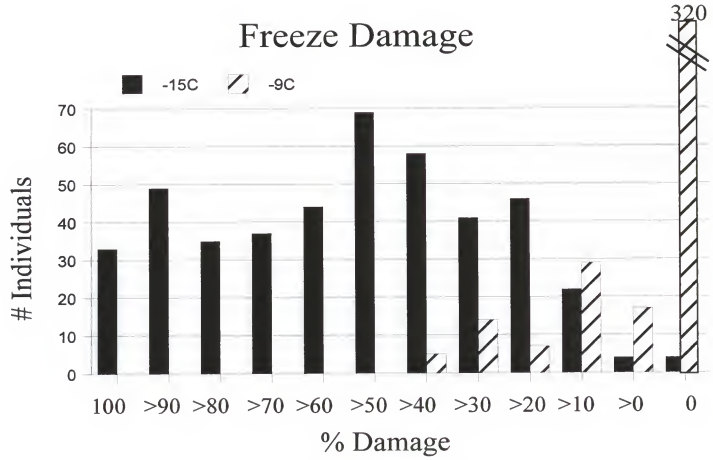

Figure 4-1: Distribution of stem dieback at -9°C and -15°C in the pseudo-testcross population.

were mapped (Figure 4-2 and 4-3). Five of these markers showed significantly different means in the bulk populations between the classes at both temperatures tested. Marker OPC02043 is located on the map near the interval located on linkage group P1, and marker OPE07100 was mapped near the intervals on linkage group P5 (Figure 4-3). Marker OPT12110 mapped to linkage group P9 (Figure 4-3). Marker A01058 is located in the interval located on the linkage group C1 (Figure 4-2) and marker OPF05019 on group C3 (Figure 4-2).

Table 4-1: RAPD markers identified using bulk segregant analysis showing significant mean stem dieback differences between the present and null classes.

Primer	Marker Size (bases)	% Mean Dieback -9°C	% Mean Dieback -15°C	Linkage Group	Parental Origination
		Marker Present/Null	Marker Present/Null		
OPA01[a]	580	4.6/24.6**	19.5/51.3*	1	*Citrus*
OPC02 [a]	430	26.8/6.4**	57.1/20.0*	1	*Poncirus*
OPE03	1100	8.3/22.1*	23.3/45.0[ns]	unassigned	*Poncirus*
OPE07	1000	28.7/11.4**	63.1/28.0*	5	*Poncirus*
OPE15	2036	26.3/8.6**	48.4/22.4[ns]	unassigned	*Citrus*
OPE19	2100	26.0/8.9*	60.1/21.0**	unassigned	*Citrus*
OPF05	190	6.3/20.0*	15.5/46.3*	3	*Citrus*
OPT12	1100	10.5/25.8*	28.3/53.9[ns]	9	*Poncirus*

[a] Also significantly different mean stem dieback between classes in the subpopulations used for mapping.

* Significantly different at $P \leq 0.05$.

** Significantly different at $P \leq 0.01$.

ns not significantly different at $P \leq 0.05$.

Discussion

Utilizing more than one method of analyzing data has become common practice in QTL mapping. For example with pea, QTL affecting seed weight were located using interval mapping, bulked segregant analysis, and selective genotyping (Timmerman-Vaughan et al. 1996). Analysis of regression coefficients and interval mapping were used in combination to locate QTL associated with resistance to stalk and ear rot in maize (Pe et al. 1993). William et al. (1997) used bulked segregant and one-way analysis of variance to locate QTL associated with leaf rust resistance in bread wheat. With soybean, one-way ANOVA and interval mapping were used in conjunction to investigate QTL controlling reproductive, morphological, and seed traits (Mansur et al. 1993). Analyzing data with more than one method one can raise the level of confidence in QTL located for a particular trait.

For this population of *Citrus grandis* x *Poncirus trifoliata,* three intervals with marginal LOD scores were located. Their validity was corroborated with results from means comparison, one-way analysis of variance, and bulked segregant analysis. The interval identified on the linkage group C1 was identified using two different QTL mapping programs and was associated with markers identified through means comparison, one-way ANOVA, and bulked segregant analysis (Figure 4-2).The interval on linkage group C5 was associated with markers identified through means comparisons and one-way ANOVA (Figure 4-2).

The major QTL interval region on the *Poncirus* map was further confirmed by analysis with other methods. The intervals located on P5 were identified using both QTL

mapping programs and also were associated with markers identified through all other means. The interval on linkage group P1 was associated with markers identified using means comparison, one-way ANOVA, and bulked segregant analysis also.

In addition, two regions, one on each map, were not identified through interval mapping but were identified through means comparison and one-way ANOVA. By utilizing multiple analysis methods one can increase the confidence in putative QTL located and decrease the odds of missing significant QTL associated with the trait being studied.

The mapping of QTL in this population indicates there is one major QTL or group of QTL located in *Poncirus* that has a large effect on the level of cold tolerance seen. In addition 3-5 or more QTL contribute a smaller effect on the level of tolerance. In hybrid populations of *Poncirus* with various *Citrus* species, the level of cold tolerance remains high in some individuals in advanced populations even as the percentage of *Poncirus* parentage is reduced (Barrett 1981). This could indicate a major gene effect which corresponds to a major QTL controlling a large portion of the variance for cold tolerance.

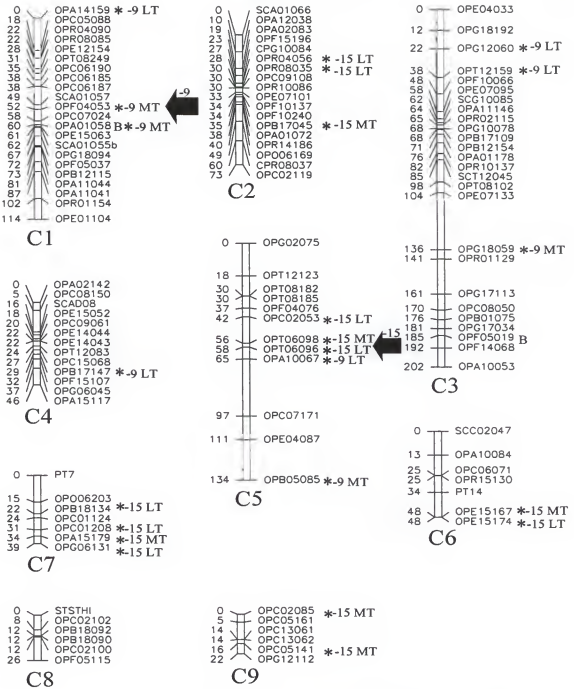

Figure 4-2: Linkage map of *Citrus grandis* showing QTL identified with multiple methods. Areas identified by interval analysis are indicated with arrows with the temperature for which significance was indicated. Markers identified through bulked segregant analysis are identified with a large B and those through mean comparison or ANOVA with an asterisk (*), the temperature at which the difference was found, and whether presence of the marker indicated more tolerance (MT) or less tolerance (LT).

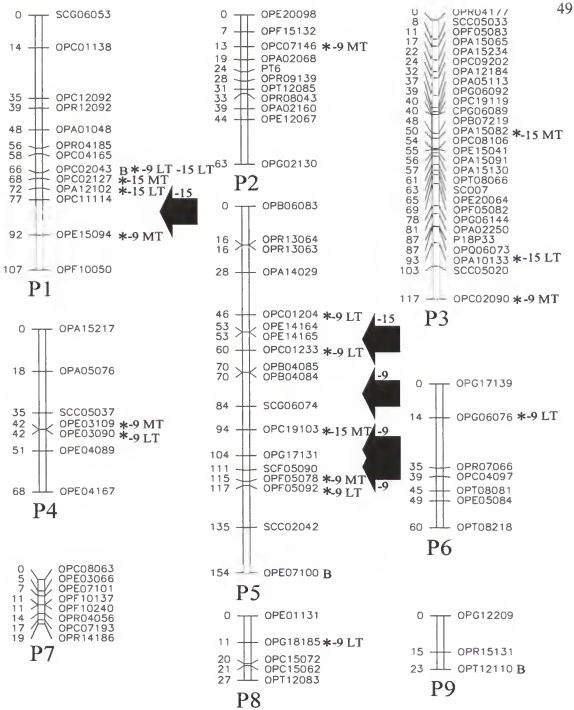

Figure 4-3: Linkage map of *Poncirus trifoliata* showing QTL identified with multiple methods. Areas identified by interval analysis are indicated with arrows and the temperature for which significance was indicated. Markers identified through bulked segregant analysis are identified with a large B and those through mean comparison or ANOVA with an asterisk (*), the temperature at which the difference was found, and whether presence of the marker indicated more tolerance (MT) or less tolerance (LT).

CHAPTER 5
EVALUATION OF COLINEARITY BETWEEN THE *Citrus grandis* 'DPI 6-4' x *Poncirus trifoliata* 'Rubideaux' F_1 PSEUDO-TESTCROSS AND THE *Citrus grandis* 'Thong Dee' x (*Citrus grandis* 'Thong Dee' x *Poncirus trifoliata* 'Pomeroy') BC_1 POPULATION MAPS WITH SEQUENCE BASED PCR MARKERS

Introduction

Molecular markers have made mapping genomes of diverse species possible by making the generation of large numbers of segregating markers a reality. RAPD markers have become the choice of many researchers to begin genetic mapping in species that are difficult to map due to biological vagaries such as long juvenile/generation times, large plant size, self-incompatibility, and reproductive irregularities such as apomixis or seedlessness. RAPD markers have been to used to produce maps for difficult species that display some or all of these characteristics including *Citrus* (Cai et al. 1994), apple (Maliepaard et al. 1998), grape (Lodhi et al.1995), blueberry (Qu and Hancock 1997) and oilseed rape (Hansen et al. 1997). To be able to integrate genetic maps produced from different populations in order to fully utilize the genetic information available, a minimum number of common markers must be available (Stam 1993). However, RAPD markers exhibit several flaws that make them unreliable for colinearity and homology analysis between related species and populations. First, most RAPD markers show dominant inheritance, which makes the information gleaned from each marker less than

that obtained from markers with co-dominant inheritance. Also, the primers used to produce RAPD markers are usually only 10 bases long so that multiple marker loci are often produced with each primer. This can lead to unreliable results and scoring in reactions using different populations or done in different laboratories with different equipment. These marker loci are usually not genetically linked. Therefore, co-dominant markers that are locus specific would be desirable to evaluate homology and colinearity between genetic maps of different populations and species.

Several types of molecular markers displaying co-dominant inheritance have been developed that show a high degree of loci specificity and reliability between related populations and species. Restriction fragment-length polymorphisms (RFLPs) are the most commonly used markers with these qualities. Maps of wheat and barley (Dubcovsky et al. 1996) and of foxtail millet and rice (Devos et al. 1998) were analyzed for colinearity and regions of homology using RFLP markers. Analysis of wide crosses between species of tomato (Grandillo and Tanksley 1996) and wheat (Cadalen et al. 1997) has been performed using RFLP markers and, in apple, male and female linkage maps were aligned using RFLPs and other markers (Maliepaard 1998).

RFLP markers are not ideal for this procedure because of the technical difficulty involved in preparing radioactive probes and scoring large populations. Large amounts of DNA in the microgram range are needed from each individual in the population, which is time consuming to collect, restrict, electrophorese, blot, and probe for polymorphism. Markers based on PCR technology that could be quickly screened on agarose or polyacrylamide gels and require minute amounts of DNA in the nanogram range would

be useful for comparisons of genetic maps. Many such marker types have been developed, including sequence-characterized amplified regions (SCAR) (Paran and Michelmore 1993), cleaved-amplified polymorphic sequences (CAPS) (Konieczny and Ausubel 1993), and sequence-tagged sites (STS). All of these markers use a pair of forward and reverse primers specific to one DNA sequence. Multiple amplification products can be seen with these primers if the DNA sequences targeted are duplicated or highly repetitive.

SCAR markers are derived by cloning and sequencing RAPD markers so that primers specific to the sequence can be designed (Paran and Michelmore 1993). By making primers longer than the underlying RAPD marker, one increases the odds against a random primer-pair match at a region of DNA sequence other than the sequence for which the primers were designed. SCAR markers can be either dominantly or co-dominantly inherited depending on whether the underlying cause of the polymorphism is a primer site difference or an amplified-product length difference.

CAPS can be produced from many types of DNA sequence including cloned RAPDs, expressed-sequence tags (ESTs), sequenced RFLP probes, and genomic DNA clones (Staub 1996). Single-nucleotide polymorphisms between marker alleles that alter restriction-enzyme target sites enable the different alleles to be identified because of differential restriction (Konieczny and Ausubel 1993). CAPS have been produced from sequences intended for SCAR markers that did not show polymorphism (Ohmori et al. 1996, Deng et al. 1997), EST sequences (Gilpin et al. 1997, Botella et al. 1997), and genomic clones (Hardtke et al. 1996).

STS markers are most commonly derived from sequenced RFLP probes (Blair and McCouch 1997; Larson et al., 1996) or from EST sequences (Gilpin et al. 1997). They are often similar to CAPS markers in the need for digestion for identification of polymorphisms, but size differentiation is sometimes detected without digestion, similar to co-dominant SCAR markers.

In this manuscript, all markers derived from cloned and sequenced RAPD markers will be referred to as SCARs if polymorphism was found without restriction and as CAPS if restriction was needed to identify polymorphism. Also, markers derived from EST sequences without the need for digestion will be termed STS markers. These three types of markers were used to establish common markers between the two maps for the *Citrus grandis* 'DPI 6-4' x *Poncirus trifoliata* 'Rubideaux' F_1 pseudo-testcross population and one map of the *Citrus grandis* 'Thong Dee' x (*Citrus grandis* 'Thong Dee' x *Poncirus trifoliata* 'Pomeroy') BC_1 population. Alignment of these maps was done based on the location of these markers, and any RAPD markers with bright bands identified as likely common markers that corroborate the position of the other marker types. In addition, by using the BC_1 population map as an intermediary, it was possible to pair some of the homologous chromosomes from the *Citrus* and *Poncirus* maps constructed from the pseudo-testcross population. Pairing of homologous chromosomes in apple was done using RFLP markers that were heterozygous in both parents and for which the each parental allele could be followed (Hemmet et al. 1994). However, because of the wide divergence between the parents in the *Citrus* x *Poncirus* pseudo-testcross, no markers of this type were available in this population. This made the use of the BC_1 population map

as an intermediary, which contains markers alleles originating from both *Citrus* and *Poncirus,* an alternative option.

Materials and Methods

Plant Material

The *Citrus grandis* 'DPI 6-4' x *Poncirus trifoliata* 'Rubideaux' F_1 pseudo-testcross population used for mapping with RAPD markers was used to screen sequence-based PCR primer-pairs for polymorphisms. The same sixty one individuals used for RAPD mapping were scored for these markers. Markers identified were also screened in the *Citrus grandis* 'Thong Dee' x (*Citrus grandis* 'Thong Dee' x *Poncirus trifoliata* 'Pomeroy') BC_1 population to place them on the map published by Cai et al. (1994) and Durham et al. (1992). The parents and only 44 individuals from this population were scored due to difficulties in obtaining plant material for DNA isolation for some of the progeny.

DNA Isolation

Genomic DNA was isolated as described by Durham et al. (1992) and modified for 1.5 mL microfuge tubes, as described in Chapter 3. Young shoot material was used when possible for highest yields but mature leaf material produced adequate yields when necessary. The DNA concentration was determined using a spectrophotometer, and a diluted DNA stock of 10 ng/μl was made for DNA amplification.

Cloning and Sequencing RAPD Products

Two methods of shotgun cloning were utilized either following the protocol of Comes et al. (1997) to clone entire RAPD profiles with multiple polymorphic loci for screening or modifying this procedure to target particularly desirable bands in RAPD profiles such as very bright bands or co-dominant RAPD markers. In the unmodified method, the amplified products were isolated directly from PCR reactions using a QIAquick PCR purification kit (Qiagen, Inc.) following the manufacturer's protocol. The modified isolation procedure utilized the solubility of DNA in water to isolate one or more bands from individual gel profiles. Using a wide bore pipette tip, agarose plugs containing the desired DNA band were placed overnight at 4°C in the volume of water needed for PCR reactions so that the DNA could leach from the agarose into the water. The water with the DNA was then used in a PCR reaction to reamplify the desired bands. These were then isolated directly from the PCR reaction using a QIAquick PCR purification kit (Qiagen, Inc.).

Cloning was done using the pGEM-T Easy Vector System (Promega Corp.) following the manufacturer's protocols. Five micro liters of isolated DNA solution was used in each cloning reaction. Quantifying the DNA concentration before cloning was difficult and unreliable due to the small size of the DNA fragments isolated and proved unnecessary for successful cloning. Fragments were ligated immediately following isolation into the pGEM-T Easy vector. The pGEM-T Easy vector has a thymine base overhang at the cut ends that complements the adenine overhang on fragments produced in PCR with Taq polymerase (Mezei and Storts 1994). Care must be taken to ligate within

hours of the end of the PCR reaction, as the adenine overhang degrades with time which reduces ligation efficiency.

E. coli DH5α competent cells (Gibco BRL Products, Life Technologies) were transformed using the following protocol. Competent cells were thawed on ice for 20 minutes and then gently mixed with a wide bore pipette tip. A 100 μl volume of cells was dispensed into a 1.5 ml micro-centrifuge tube on ice. To that was added 2μl of the ligation reaction, and the mixture was gently swirled before incubation on ice for 30 minutes. Cells were heat shocked by immersion in a 42°C water bath for 55 seconds. Then 400 μl of room temperature LB medium was added and mixed gently. The cells were spread on LB medium plates containing 1.5% Bacto agar, 100 μg/ml ampicillin, 0.5 mM IPTG, and 80 μg/ml X-gal for blue/white screening. The cells were incubated at 37°C overnight. Stabs of white colonies were screened with PCR by directly inserting the cells into PCR reactions using SP6 and T7 primers complementary to regions flanking the vector insert site to identify clones desirable for sequencing. Each 50 μl reaction consisted of buffer [50mM KCl, 10 mM Tris-HCl (pH 9.0), 1% Triton X-100] (Promega), 2 mM $MgCl_2$, 0.8 mM dNTPs (200 μM of each dNTP), 0.4 μM of each primer, 2 U of Taq polymerase (Promega), a colony sample for the DNA template, and water to volume. Amplification was done in an MJ PTC-100™ thermocontroller with 96 wells and a heated lid (MJ Research, Inc.). The following conditions were used: 1 minute at 93°C, 1 minute at 55°C, and 2 minutes at 72°C for 42 cycles. A final extension step at 72°C for 10 minutes concluded the amplification. The amplification products were separated in

2% agarose gels with TAE buffer (Sambrook et al. 1989) by electrophoresis in a Wide Mini-Sub™ cell (Bio-Rad) and detected by ethidium bromide staining.

Colonies with desired inserts were streaked again on LB/ampicillin/IPTG/X-gal plates and grown overnight. Each target insert was amplified in two 50 μl reactions for purification and sequencing. Sequencing was done by the University of Florida's ICBR DNA Sequencing Core Lab.

SCAR and CAP Primer Design and Amplification Protocol

Based upon the sequence of the cloned RAPD fragments, primers were designed ranging in size from 18 to 27 bases for specific amplification of the fragments. The forward and reverse primers each contained the original 10 base Operon Technologies RAPD primer followed by additional bases specific for both of the complementary strands of DNA sequence. Care was taken to make the melting point of each primer pair to be roughly equal and between 58°C and 70°C with minimal internal structure. Whenever possible, the primers were ended with a guanine or cytosine base to improve annealing.

The 50 μl amplification reactions for the tree DNA used the above procedure except for 50 ng of genomic DNA was used instead of bacterial stabs. For primer pairs producing faint bands, the annealing was lowered to 45°C to try to improve amplification. For pairs producing multiple bands, the annealing temperature was raised to 60°C to increase specificity. The amplification products were visualized as above.

CAPS Production Protocol

SCAR primer pairs, that showed polymorphism between the parents but not in a small subset of progeny, were targeted for CAPS marker production. These amplified products were digested with 4 or more of the following restriction enzymes: Rsa I, Alu I, Taq I, Hpa II, Tru9 I, Hae III, Hinf I, Cfo I, and EcoR V (Promega). Each digestion contained 5 μl of PCR reaction, 1.5 μl of the corresponding 10x restriction buffer (Promega), 0.15 μl of 10 mg/ml bovine serum albumin (Promega), 0.3 μl of restriction enzyme and 8.05 μl of water to bring the volume to 15 μl. The reactions were then incubated at 37°C for 1.5 hours in a MJ PTC-100™ thermocontrollers with 96 wells (MJ Research, Inc.), except when Taq I or Tru9 I was used for digestion in which case the were incubated at 65°C. The digestion products were separated as described above.

STS Primer Design and Production

Citrus EST sequences were obtained from the Genbank EST database for candidate sequences for STS marker production. Ten sequences were chosen for primer design that were greater than 500 bases long, and that were rich in guanine (G) and cytosine (C) with low levels of repeats. Forward and reverse primers were designed between 18 and 23 bases long with 60-70% GC content from conserved regions of the sequence if possible with low secondary structure and primer dimer capability. Fragment amplification and visualization was done in the same manner as for the SCAR markers. Non-polymorphic amplification products were subjected to the same protocol as the CAPS markers to try to find polymorphisms.

Genetic Mapping

Mapping was done with the JoinMap computer program (Stam 1993) to place the sequence-based PCR markers on the linkage groups produced with RAPD markers in the *Citrus grandis* 'DPI 6-4' x *Poncirus trifoliata* 'Rubideaux' F_1 pseudo-testcross population. The same was done using data for the *Citrus grandis* 'Thong Dee' x (*Citrus grandis* 'Thong Dee' x *Poncirus trifoliata* 'Pomeroy') BC_1 population to place them on the map published by Cai et al. (1994) and Durham et al. (1992). The map displayed for the BC_1 population has a subset of markers identified for ease of drawing but map distances were calculated using the complete data set for this population. The maps were aligned using markers common between the populations.

Results

Complete sequence was obtained and forward and reverse primers designed for 67 RAPD fragments to identify polymorphic SCAR and CAPS markers (Table 5-1). Primer pairs were also made for three incomplete sequences because both ends of the fragment sequence were available for primer design (Table 5-1). Seven sets of primers amplified no fragments under standard SCAR-PCR protocols and 8 pairs produced fragments that showed weakly in ethidium bromide stained agarose gels. Fifteen SCAR markers were produced with 9 dominantly inherited and 6 co-dominantly inherited (Figure 5-1) in the pseudo-testcross population (Table 5-2). In addition, 3 co-dominant CAPS markers were identified using restriction digestion (Figure 5-2; Table 5-3). One STS marker was identified from a *Citrus sinensis* EST homologous to a thiamine-biosynthesis gene. In

addition, 5 SCAR and 11 CAPS markers developed for the BC_1 population in the laboratory of Fred Gmitter at the University of Florida, Citrus Research and Education Center, Lake Alfred, Florida, were tested (Table 5-4). Two of the SCAR primer sets produced dominant markers in the pseudo-testcross population. Five CAPS markers proved polymorphic in the pseudo-testcross population. Thus, a total of 26 sequence based markers were identified that were polymorphic in the pseudo-testcross population. Thirteen of these were mapped to 7 different linkage groups on the *Citrus* parent map (Figure 5-3) and 11 were mapped to 5 linkage groups on the *Poncirus* parent map (Figure 5-4).

In the BC_1 population, 20 of these markers produced polymorphic fragments and 15 of these were mapped to 7 different linkage groups (Figure 5-5). Thirteen of these were in common with markers on the pseudo-testcross population maps (Table 5-6). These markers were use to align regions of the pseudo-testcross maps with the BC_1 map.

Linkage group P3 aligned with the beginning of group BC1 with 2 CAPS and 2 strong RAPD markers in common. Linkage group C4 aligned with BC1 with one CAPS marker and 1 RAPD marker. Linkage group P1 aligned with the middle of group BC1 with one SCAR marker. Linkage group C1 had one SCAR marker in common with the end of linkage group BC2 and linkage group P4 had one SCAR marker in common with the end of group BC1. Group C6 had one CAPS and 1 RAPD marker in common with the middle of group BC4. Linkage group C7 had one SCAR marker in common with the end of group BC6 and group C2 had one CAPS marker in common in the middle of BC6. Linkage group P5 aligned with group BC6 with one SCAR and one RAPD marker in

common. Linkage group C3 had one SCAR marker in common with group BC7. Linkage groups P2 and P5 had one CAPS and one SCAR marker in common respectively with group BC8 (Figure 5-6).

There were two instances where linkage groups on the backcross map showed homology between linkage groups on both of the pseudo-testcross maps, indicating a possible homologous pair of chromosomes in the pseudo-testcross population. Linkage group BC1 had markers in common with groups C4, P1, and P3. Linkage group BC6 had common markers with P5, C7, and C2.

Discussion

Sequence based PCR markers can be useful for genetic linkage mapping in multiple related populations. They are technically easier and much quicker to score in a population than RFLP and microsatellite markers. Markers produced with specific primer pairs were mapped to 12 of 18 linkage groups in the parental linkage maps in the pseudo-testcross population. The efficiency of polymorphisms identified was encouraging with 19 of 80 (24%) primer pairs tested producing SCAR, CAPS and STS markers. Of the primer pairs tested 15 of 80 (19%) produced no fragment or weak signatures. Thus 46 of 80 (58%) primer pairs produced fragments for which no polymorphisms were found in the pseudo-testcross population. Evidently, many RAPD marker polymorphisms are due to mismatches in the short 10 base primer site resulting in no amplification in some individuals. When longer, more specific primers are designed, single base mismatches in the original RAPD primer are overcome by the longer primer allowing amplification thus

eliminating the polymorphism. Paran and Michelmore (1993) found that 5 of 8 primer pairs for SCAR production were from RAPD polymorphisms apparently due to mismatches at the RAPD primer sites. Fang et al. (1997) found primers designed from RAPD marker sequences lost the polymorphism when used for SCAR marker production. Xu et al. (1995) also experienced loss of polymorphism when designing primers from RAPD marker sequences. Markussen et al. (1995) also found that the specificity of SCAR-marker primer sets was not always at a high level presumably due to duplicated or repetitive DNA sequences. They found more than one segregating marker locus with one primer pair.

Digestion with restriction enzymes can sometimes be used to locate polymorphisms due to sequence differences at restriction-enzyme cut sites. However, the efficiency of finding these type of sequence differences is low. Screening fragments in the population for restriction site polymorphisms is labor intensive and time consuming with no guarantee of success. Fang et al. (1997) were unable to find a cut site polymorphism in a fragment produced from primers designed from RAPD marker sequence. With non-polymorphic SCAR fragments in grape, Xu et al. (1995) were unable to find restriction site polymorphisms even when digested with 7 different restriction enzymes.

Thus, producing markers based upon RAPD product sequences can prove to be difficult but relatively efficient when compared to RFLP markers. Similarly EST sequences also contain low levels of polymorphism for both size and restriction site differences. A relatively large number of primer-specific PCR markers were developed in a short period of time in relation to the time needed to identify a like number of RFLP

markers. Also, these markers can be transferred between populations like RFLP markers. Of 26 polymorphic markers useful in the pseudo-testcross population, 20 (77%) were also useful in the BC_1 population. In addition, 13 of 20 (65%) markers polymorphic in both populations were mapped and could be used for map alignment. Also, two likely pairs of homologous chromosomes were identified in the pseudo-testcross population by utilizing the common sequence based PCR markers with the BC_1 map as an intermediary. Linkage group P3 is likely homologous to group C4 and linkage group P5 is likely homologous to both C7 and C2. As more sequence based PCR markers are added to the maps more homologous chromosome pairs should be identified.

Thus, sequence-based PCR markers can be produced and used successfully from RAPD and EST-database sequences. These types of markers may be especially useful for targeting specific regions of the genome or specific genes of interest rather than map coverage. Parent and Page (1998) used SCAR markers for identification of raspberry cultivars. Deng et al. (1997) used SCAR and CAPS markers to target the gene for resistance to citrus tristeza virus for use in marker assisted selection. The ease in scoring these types of markers can make them useful for marker assisted selection in breeding programs when specific genes or genomic regions are targeted.

Table 5-1: EST sequences and specific primers tested for STS marker production in the F_1 pseudo-testcross population.

EST sequence name	Specific primers (5' to 3')	Target fragment size*
similar to fimbriata associated protein	GGCGACCCAGGATAGCAAAC GGACCAGACCAACTGCTACC	489
isoflavone reductase-like protein	GGGGGTACTGGATACTTCGG CAAAAGCCGTACGTGCAGGC	919
thiamine biosynthesis homologous gene	CTTCTAAGCAGCAGGGGAAGG CCCATTGGCTGATGCCTCAAC	1448
hypothetical protein-wound inducible	CGACGCTTCCTTCATGCTGTG CGTAACAGGCATATCGGTGGC	934
acid invertase gene	GGTGACATAGTGTGGGGG GTGGCGTAGTTCTTCCCG	674
sucrose phosphate synthase gene	GCTTGCCCGGGCACTTGCAAG CCTTAGGGGCCGACACTCTCC	932
phenylalanine ammonia-lysase mRNA	GGTTGTGCACAGGTACTGACC GGGAAGAGGAGCACCATTCC	1058
blight induced cDNA-superoxide dismutase	GCGGTGGCGGTGATGACGGG GTCCGCACGACACGTGACAGG	561
beta-1,3-glucanase gene	CCCTTCAATGGCTCCCGTCG CAGAGCCGGCCTTGGAAACC	443
similar to B2 protein in carrot	GACTTGACAGTATTGGCTGGC CACTTTCACATCTTGCAAGTCTG	1197

* Fragment sizes are in number of nucleotide bases and are not as long as the EST sequence.

SCF05090

Figure 5-1: Example of a SCAR marker. The first lane contains a DNA ladder of known size for comparison to amplified fragment size. The second lane is the *Citrus* parent and the third is the *Poncirus* parent. The rest of the lanes are progeny DNA fragments. Note that the *Poncirus* parent contains two marker alleles and each of the progeny contains one or the other. The *Citrus* parent is null for this marker.

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Figure 5-2: Example of a CAPS marker. The first lane contains a DNA ladder of known size for comparison to amplified fragment size. The second lane is the *Citrus* parent and the third is the *Poncirus* parent. The rest of the lanes are progeny DNA fragments. Note that the *Poncirus* parent contains two marker alleles. One is cut and one is uncut. Each of the progeny contains one or the other. The *Citrus* parent shows only an uncut fragment.

Table 5-2: Forward and reverse primers for cloned RAPD fragments tested for SCAR and CAPS markers in the F_1 pseudo-testcross population with size indicated in nucleotide bases.

RAPD Primer Name (Operon)	Specific Primer (RAPD Primer underlined) (5' to 3')	Fragment Size (bases)
OPA01	CAGGCCCTTCTTATGTCATC CAGGCCCTTCACCAAACACG	571
OPA01	CAGGCCCTTCATGTTGAGAG CAGGCCCTTCTCCGGGAAAG	661
OPA01	CAGGCCCTTCAACCAGAGCT CAGGCCCTTCCTCAGGTTTG	623
OPA01	CAGGCCCTTCCTCGTTAACTA CAGGCCCTTCGCAAAGATAAC	554
OPA01	CAGGCCCTTCACCAACACGAAC CAGGCCCTTCTTATGTCATCAAAG	545
OPA15	TTCCGAACCCTCTCTTTAAAATTC TTCCGAACCCCCTTTACTTG	461
OPA15	TTCCGAACCCTGCTGTAACC TTCCGAACCCAATGCCCTTG	736
OPA15	TTCCGAACCCTACTTTACTC TTCCGAACCCTAGCGAGTTTTGC	881
OPB07	GGTGACGCAGGATCATTGCC GGTGACGCAGAGGCACGCAC	387
OPB07	GGTGACGCAGCAGCATATCAG GGTGACGCAGTTTGACCACG	135
OPB17	AGGGAACGAGCATTTTCGGG AGGGAACGAGGGGAAGAATC	467
OPB18	CCACAGCAGTGATAAAATTAC CCACAGCAGTTTAGACACCC	692
OPC02	GTGAGGCGTCATTGATCCA GTGAGGCGTCTTCATAGAC	485
OPC02	GTGAGGCGTCCTCGAACCAG GTGAGGCGTCAACTCTAATCG	415

Table 5-2: cont.

RAPD Primer Name (Operon)	Specific Primer (RAPD Primer underlined) (5' to 3')	Fragment Size (bases)
OPC02	GTGAGGCGTCAATTCAAAGAAG GTGAGGCGTCCAATGACCTC	468
OPC02	GTGAGGCGTCGCCGAGAGG GTGAGGCGTCCAATGGGTTG	342
OPC02	GTGAGGCGTCATCCCCGTC GTGAGGCGTCGTACACAAGAC	392
OPC05	GATGACCGCCGCTTTATCCAAG GATGACCGCCCTAGGAAATGG	648
OPC05	GATGACCGCCCCGTCCAAGAG GATGACCGCCCTCGCTTCCTC	325
OPC05	GATGACCGCCATAGGTCCGAG GATGACCGCCGGGACAAAAATC	367
OPC05	GATGACCGCCTTCGTGATGC GATGACCGCCCACAAAGCTG	199
OPC05	GATGACCGCCAGGCGATAGC GATGACCGCCAAACAGGCC	367
OPC08	TGGACCGGTGAAGGCAGCC TGGACCGGTGGATGAGTAGG	>1160*
OPC08	TGGACCGGTGGGTAAAAGCAAG TGGACCGGTGCAGGTAGAGAAC	731
OPC08	TGGACCGGTGCTGCTGCGAC TGGACCGGTGGACAATGGAG	567
OPC15	GACGGATCAGCATTTAGCAAC GACGGATCAGGTGTGTTGAAC	534
OPC15	GACGGATCAGATGGATTAAGTG GACGGATCAGGTCAGGGAAG	252
OPE03	CCAGATGCACCGCACACAAC CCAGATGCACACTCTAAAATC	500

Table 5-2: cont.

RAPD Primer Name (Operon)	Specific Primer (RAPD Primer underlined) (5' to 3')	Fragment Size (bases)
OPE03	CCAGATGCACCCTTACAAATC CCAGATGCACGTACAACAAG	428
OPE07	AGATGCAGCCTAACACATTATC AGATGCAGCCATTGAACGCTC	705
OPE07	AGATGCAGCCAACCAACCATC AGATGCAGCCCCTTCATGTAAG	553
OPE07	AGATGCAGCCAACACATTATCGG AGATGCAGCCATTGAACGCTCC	713
OPE15	ACGCACAACCCGCACAGAAG ACGCACAACCACCAACACAAC	420
OPE15	ACGCACAACCCTATTACATGCAG ACGCACAACCAGAAGGCATTG	383
OPE15	ACGCACAACCTCGTAACATATC ACGCACAACCCCATATTCAC	542
OPE15	ACGCACAACCATCATCTTTCC ACGCACAACCCCAATACACC	376
OPE15	ACGCACAACCAAACATTATCGC ACGCACAACCACTGCTACTTC	610
OPE15	ACGCACAACCATATAGCAAGAG ACGCACAACCAACTTTTCATCTC	>654*
OPE15	ACGCACAACCAACTTTAAGCC ACGCACAACCCTAATACACCC	419
OPE15	ACGCACAACCGCTTTCCTAC ACGCACAACCAGATGGCATTG	531
OPE15	ACGCACAACCACCAACACAAC ACGCACAACCCGCACAGAAG	461
OPF05	CCGAATTCCCCACGTTATTTAC CCGAATTCCCAGTCAAATTCC	247

Table 5-2: cont.

RAPD Primer Name (Operon)	Specific Primer (RAPD Primer underlined) (5' to 3')	Fragment Size (bases)
OPF05	CCGAATTCCCAGACGTAAAGC CCGAATTCCCATAAATTGATGTG	903
OPF05	CCGAATTCCCTATAAGGCCC CCGAATTCCCATTTCCAAGAT	336
OPF05	CCGAATTCCCGTAAAAGTAAC CCGAATTCCCCATGTTATTTAC	225
OPG06	GTGCCTAACCACTACTGCAG GTGCCTAACCTAGGTTGCTAC	897
OPG06	GTGCCTAACCCCTGAGTACTTTTG GTGCCTAACCTCTGGATGAATG	>739*
OPG06	GTGCCTAACCCAGTGTCTGG GTGCCTAACCTCACGAACACC	530
OPG06	GTGCCTAACCACTACTGCAGAG GTGCCTAACCTACCTTGCTAC	891
OPG06	GTGCCTAACCCATGTCGGTG GTGCCTAACCTCAAGTTTGC	216
OPG06	GTGCCTAACCTGGATCCCATTTATG GTGCCTAACCTCCCGGCGAAG	1018
OPG10	AGGGCCGTCTATGGATTCATGC AGGGCCGTCTCACTTGAAATTGG	840
OPG10	AGGGCCGTCTAGGAGTTCTTAG AGGGCCGTCTAGAAACCCCAG	413
OPG10	AGGGCCGTCTATATGACGTGAG AGGGCCGTCTCCAACTTCACATG	851
OPG10	AGGGCCGTCTCTCNCAAGTTTC AGGGCCGTCTACTTGGCCTGC	533
OPQ06	GAGCGCCTTGTGGAAAATTGC GAGCGCCTTGACGGGTTTTAAC	633

Table 5-2: cont.

RAPD Primer Name (Operon)	Specific Primer (RAPD Primer underlined) (5' to 3')	Fragment Size (bases)
OPQ06	GAGCGCCTTGACAACAATATG GAGCGCCTTGAGATTCGGTTTTG	347
OPR08	CCCGTTGCCTCTATAGATGTC CCCGTTGCCTTGACCGCC	366
OPT08	AACGGCGACACTGCTCATTAG AACGGCGACAGCGGAGGTC	475
OPT08	AACGGCGACAAGAAATGATTGG AACGGCGACAATAGCGGAGG	330
OPT08	AACGGCGACAGCTGTTTCCAT AACGGCGACACATTAACAAAAC	219
OPT08	AACGGCGACAGCGGAGGTCC AACGGCGACACTGCTCATTAG	494
OPT08	AACGGCGACATCACGATGGG AACGGCGACACATGCCCGCA	324
OPT08	AACGGCGACAACACAAACAC AACGGCGACAGTGTTGTTG	902
OPT08	AACGGCGACATAGACACGATC AACGGCGACAACATGAAGAAG	847
OPT12	GGGTGTGTAGAGCAATATTTTAAC GGGTGTGTAGTTAAGCTTTAC	604
OPT12	GGGTGTGTAGTGAGTGAGAG GGGTGTGTAGGGTAATAATAAG	822
OPT12	GGGTGTGTAGAACGAGAGAG GGGTGTGTAGTCAATCTCTC	186
OPT12	GGGTGTGTAGCCATATACATAC GGGTGTGTAGCATTAAAAAAATAGG	541
OPT12	GGGTGTGTAGGTGCATAAA GGGTGTGTAGCCTAAAGTC	451

* Fragment with incomplete sequence obtained.

Table 5-3: SCAR markers developed from RAPD fragments in the F_1 pseudo-testcross population. Dominant or co-dominant inheritance is indicated for each.

SCAR Name	Specific Primers (5' to 3')	Type of Inheritance	Map Position
SCA01057	CAGGCCCTTCTTATGTCATC CAGGCCCTTCACCAAACACG	co-dominant	C1
SCAO1066	CAGGCCCTTCATGTTGAGAG CAGGCCCTTCTCCGGGAAAG	dominant	C2
SCA01055	CAGGCCCTTCCTCGTTAACTA CAGGCCCTTCGCAAAGATAAC	dominant	N/A
SCA01055b	CAGGCCCTTCACCAACACGAAC CAGGCCCTTCTTATGTCATCAAAG	co-dominant	C1
SCB07039	GGTGACGCAGGATCATTGCC GGTGACGCAGAGGCACGCAC	dominant	N/A
SCC02047	GTGAGGCGTCAATTCAAAGAAG GTGAGGCGTCCAATGACCTC	dominant	C6
SCC02042	GTGAGGCGTCCTCGAACCAG GTGAGGCGTCAACTCTAATCG	dominant	P5
SCC05037	GATGACCGCCAGGCGATAGC GATGACCGCCAAACAGGCC	dominant	P4
SCC05020	GATGACCGCCTTCGTGATGC GATGACCGCCCACAAAGCTG	dominant	P3
SCC05033	GATGACCGCCCCGTCCAAGAG GATGACCGCCCTCGCTTCCTC	dominant	P3
SCF05090	CCGAATTCCCAGACGTAAAGC CCGAATTCCCATAAATTGATGTG	co-dominant	P5
SCG06053	GTGCCTAACCCAGTGTCTGG GTGCCTAACCTCACGAACACC	co-dominant	P1
SCG06074	GTGCCTAACCCCTGAGTACTTTTG GTGCCTAACCTCTGGATGAATG	co-dominant	P5

Table 5-3: cont.

SCAR Name	Specific Primers (5' to 3')	Type of Inheritance	Map Position
SCG10085	AGGGCCGTCTATATGACGTGAG AGGGCCGTCTCCAACTTCACATG	co-dominant	C3
SCT12045	GGGTGTGTAGGTGCATAAA GGGTGTGTAGCCTAAAGTC	dominant	C3

Table 5-4: CAPS markers developed from RAPD fragments in the F_1 pseudo-testcross population. Restriction enzymes tested on each fragment are indicated.

CAPS Name	Specific Primers (5' to 3')	Restriction Enzymes Tested	Polymorphic Enzyme(s)	Fragment Sizes	Map Position
CPG06089	GTGCCTAACCACTACTGCAGAG GTGCCTAACCTACCTTGCTAC	Rsa I	Rsa I	multiple	P3
CPG10084	AGGGCCGTCTATGGATTCATGC AGGGCCGTCTCACTTGAAATTGG	Alu I, Rsa I	Rsa I	550/290	C2
CPR08037	CCCGTTGCCTCTATAGATGTC CCCGTTGCCTTGACCGCC	Hae III, Alu I, Rsa I, Hpa II	Rsa I/Hpa II	190/180	C2

Table 5-5: SCAR and CAPS markers developed at the Citrus Research and Education Center in Lake Alfred, FL by F. Gmitter and Z. Deng used in the F_1 pseudo-testcross population.

Marker Name	Marker Type	Inheritance Type	Map Location
PT6	CAPS	co-dominant	P2
PT7	SCAR	dominant	C7
PT14	CAPS	co-dominant	C6
PT18	CAPS	co-dominant	N/A
18P33	CAPS	co-dominant	P3
SCAD08	CAPS	co-dominant	C4
SC007	SCAR	dominant	P3

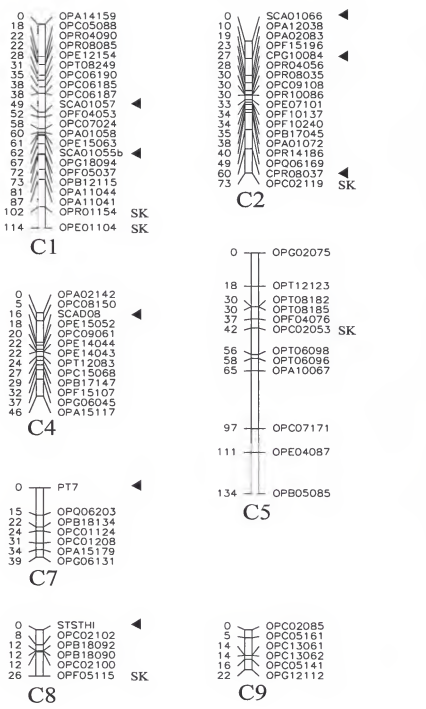

Figure 5-3: Linkage map for *Citrus grandis* 'DPI' 6-4 containing RAPD, SCAR, CAPS, and STS markers. RAPD markers are labeled beginning with OP, SCARs with SC, and CAPS with CP followed by the primer designation and size of the fragment in base pairs. STS markers are labeled with STS followed by an EST sequence designation. Each of these markers are pointed to with ▲. Skewed markers are marked with SK.

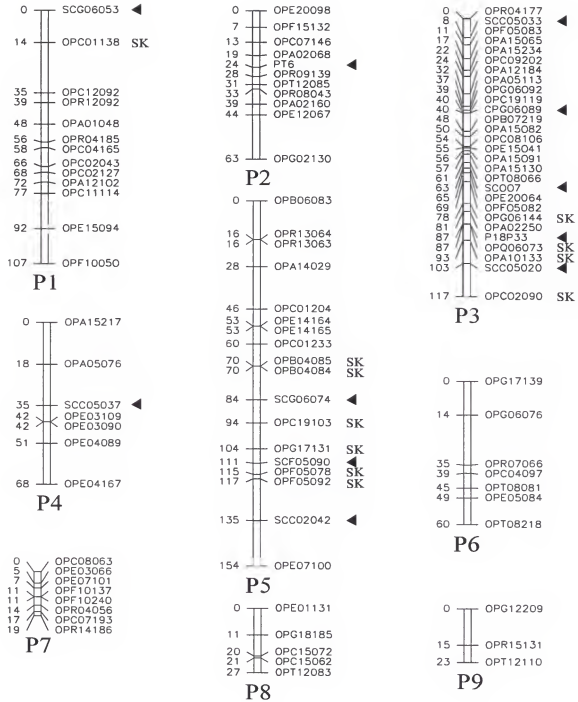

Figure 5-4: Linkage map for *Poncirus trifoliata* 'Rubideaux' containing RAPD, SCAR, and CAPS markers. RAPD markers are labeled beginning with OP, SCARs with SC, and CAPS with CP followed by the RAPD primer designation and size of the fragment in base pairs. Each of these markers are pointed to with ▲. Skewed markers are marked with SK.

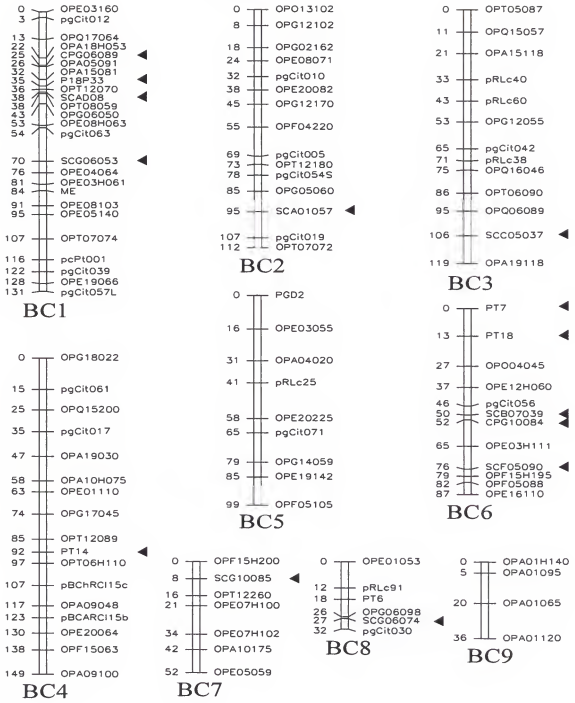

Figure 5-5: Linkage map for the *Citrus grandis* 'Thong Dee' x *(Citrus grandis* 'Thong Dee' x *Poncirus trifoliata* 'Pomeroy') BC_1 population containing RAPD, SCAR, and CAPS markers. RAPD markers are labeled beginning with OP, SCARs with SC, and CAPS with CP followed by the RAPD primer designation and size of the fragment in base pairs. Each of these are markers are pointed to with ▲.

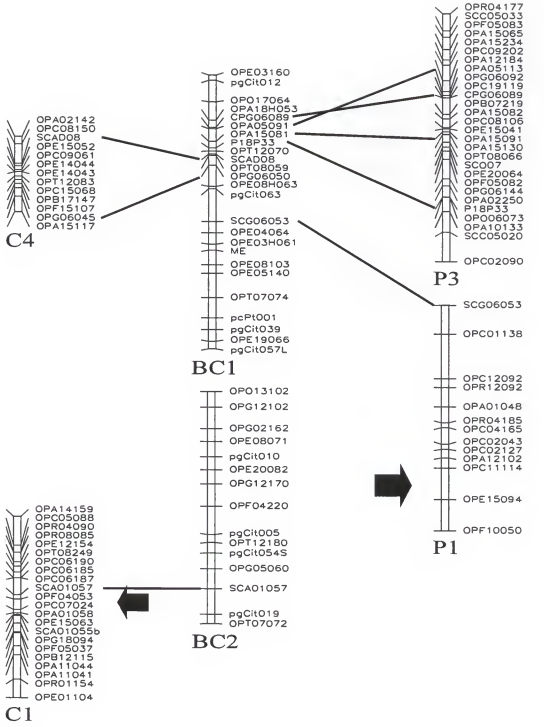

Figure 5-6:Linkage groups from the F_1, and BC_1 maps showing RAPD, SCAR, and CAPS markers in common. Lines drawn between markers indicate the colinearity between the linkage groups. Putative QTL locations are indicated with arrows.

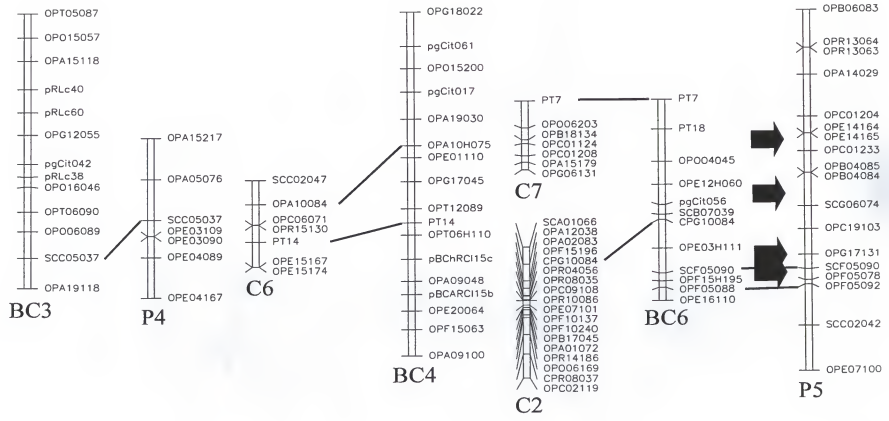

Figure 5-6: cont.

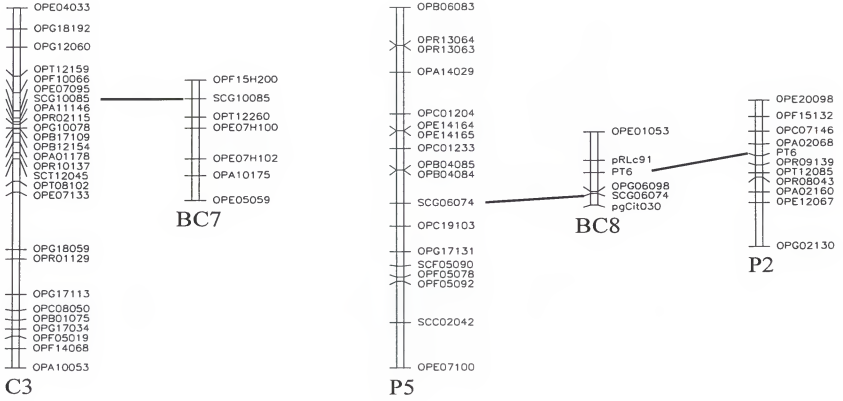

Figure 5-6: cont.

CHAPTER 6
CONCLUSIONS

Using molecular markers to map quantitative trait loci associated with cold tolerance in the wide cross between *C. grandis* 'DPI 6-4' x *P. trifoliata* 'Rubideaux' was a successful first step in elucidating the complex nature of cold tolerance in citrus trees. The pseudo-testcross population design has proven to be excellent for mapping *C. grandis* and *P. trifoliata.* Ample RAPD markers were produced to construct a linkage map of both *Poncirus* and *Citrus*. In *Citrus*, RAPDs have previously provided numerous markers for mapping throughout the genome due to the highly heterozygous nature of the genus and related species (Cai et al. 1994). The number of RAPD markers produced for this population was 4 to 5 times higher than the isozyme and RFLP markers developed for the related backcross population (Torres et al. 1985, Durham et al, 1992, Liou et al. 1996). The level of polymorphism in the pummelo parent was higher than anticipated based on the level seen for RFLP and isozyme markers in the previous studies (Torres et al. 1985, Durham et al. 1992, Liou et al. 1996).

There were a large number of RAPD markers identified that could not be placed on either of the linkage maps. This phenomenon has been seen before (Cai et al. 1994) and may be due to various causes. RAPD markers are prone to inconsistency because many of the polymorphisms are due to the single base differences that may sometimes be

allowed for in some amplification reactions thus producing spurious marker loci. Markers showing a segregation ratio skewed from the expected ratio near the end of some linkage groups may be due to recombination interference of the telomeric region of the chromosomes. Other skewed markers within the linkage groups could be a result of interference from the centromeric regions.

The *C. grandis* map is the first true map of *Citrus,* as it was produced with polymorphisms originating solely from *Citrus.* Earlier maps mostly mapped polymorphisms originating from the related species *P. trifoliata*. The maps produced in this study provide a good framework for doing QTL analysis for cold tolerance in this population. By utilizing more than one method of analyzing data a higher level of confidence of putative QTL located was achieved.

For this population of *Citrus grandis* x *Poncirus trifoliata,* three map intervals associated cold tolerance with high LOD scores were located and their validity could be corroborated with results from means comparison, one-way analysis of variance, and bulked segregant analysis. In addition, two regions, one on each map, were not identified through interval mapping, but were identified through means comparison and one-way ANOVA. By utilizing multiple analysis methods, the level of confidence in the putative QTL located was increased and the chance of overlooking significant QTL for cold tolerance was decreased.

The mapping of QTL in this population indicates there is one major QTL or group of QTL located in *Poncirus* that has a large effect on the level of cold tolerance seen. In addition, 3 or more QTL contribute a smaller effect on the level of tolerance. This could

indicate a major gene effect which corresponds to a major QTL controlling a large portion of the variance for cold tolerance.

Sequence-based PCR markers were useful for genetic linkage mapping in both related populations here. Markers produced with specific primer pairs were mapped to 12 of 18 linkage groups in the parental linkage maps in the pseudo-testcross population. The efficiency of polymorphisms identified was encouraging with 19 of 80 (24%) primer pairs tested producing SCAR, CAPS and STS markers.

Some of these markers were transferred between populations like RFLP markers. Of 26 polymorphic markers screened in the pseudo-testcross population, 20 (77%) were also useful in the BC_1 population. In addition 13 of 20 (65%) markers polymorphic in both populations were mapped and could be used for map alignment. Thus sequence-based PCR markers were produced and used successfully from RAPD and EST-database sequences.

Overall, the genetic information obtained from this population allowed for the successful mapping of a new hybrid citrus population and added to the understanding of cold tolerance inherited from *Poncirus trifoliata*. Molecular markers proved to be amenable to genetic linkage and QTL mapping. The road to a brighter future for the Florida citrus industry just got shorter.

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BIOGRAPHICAL SKETCH

Courtney Alyn Weber was born on 23 June 1968 in Cissna Park, Illinois, a small farming community. He graduated from the University of Illinois-Urbana with his Bachelor of Science in Agriculture in 1990. Courtney received his Master of Science in Horticultural Science from the University of Florida in 1994. He began his doctoral program in August 1994. Courtney has accepted an appointment as Assistant Professor at Cornell University in Ithaca, New York, and will be located at the New York State Experimental Station in Geneva, New York, beginning at the completion of this degree. The appointment is for small fruit breeding specializing in varietal development of raspberry and strawberry for the Northeastern United States. Courtney will be married to Elizabeth Bihn in Gainesville, Florida, on April 24, 1999, and he is filled with anticipation for the future.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gloria A. Moore, Chair
Professor of Horticultural Sciences

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Wayne B. Sherman
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Kenneth C. Cline
Professor of Horticultural Sciences

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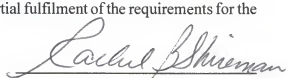
Charles L. Guy
Professor of Environmental Horticulture

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

John M. Davis
Associate Professor of Forest Resources and Conservation

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfilment of the requirements for the degree of Doctor of Philosophy.

May, 1999



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